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(21) International Application Number: PCT/US96/18197 (22) International Filing Date: 13 November 1996 (13.11.96) (30) Priority Data: <table border="0"><tr><td>60/006,619</td><td>13 November 1995 (13.11.95)</td><td>US</td></tr><tr><td>60/015,767</td><td>16 April 1996 (16.04.96)</td><td>US</td></tr><tr><td>60/020,638</td><td>27 June 1996 (27.06.96)</td><td>US</td></tr><tr><td>60/020,639</td><td>27 June 1996 (27.06.96)</td><td>US</td></tr><tr><td>60/021,965</td><td>27 June 1996 (27.06.96)</td><td>US</td></tr></table> (71)(72) Applicants and Inventors: IBANEZ, Carlos [SE/SE]; S-Stockholm (SE). ARENAS, Ernest [SE/SE]; S-Stockholm (SE). ARUMAE, Urmas [FI/FI]; FIN-Helsinki (FI). FAINZILBER, Michael [SE/SE]; S-Stockholm (SE). GRIGORIOU, Maria [GB/GB]; London (GB). KILKENNY, Carol [GB/GB]; London (GB). LINDAHL, Maria [FI/FI]; FIN-Helsinki (FI). NILSSON, Ann-Sofie [SE/SE]; S-Stockholm (SE). SAINIO, Kirsi [FI/FI]; FIN-Helsinki (FI). SALAZAR-GRUESO, Edgar [US/US]; IL (US). SARIOLA, Hannu [FI/FI]; FIN-Helsinki (FI). SIEBER, Beth-Anne [SE/SE]; S-Stockholm (SE). SUVANTO, Petro [FI/FI]; FIN-Helsinki (FI). TRUPP, Miles [SE/SE]; S-Stockholm (SE). SAARMA, Mart [FI/FI]; FIN-Helsinki (FI).		60/006,619	13 November 1995 (13.11.95)	US	60/015,767	16 April 1996 (16.04.96)	US	60/020,638	27 June 1996 (27.06.96)	US	60/020,639	27 June 1996 (27.06.96)	US	60/021,965	27 June 1996 (27.06.96)	US	(74) Agents: MILLER, Suzanne. E. et al.; Woodcock Washburn Kurtz Mackiewicz & Norris, 46th floor, One Liberty Place, Philadelphia, PA 19103 (US). (81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
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(54) Title: GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR RECEPTORS

(57) Abstract

Receptors for Glial Cell Line-Derived Neurotrophic Factor (GDNF), their cellular expression, isolation, and biochemical characterization are disclosed. C-RET is disclosed as one receptor for GDNF; additional novel receptors are also disclosed. The preparation of monoclonal antibodies directed against GDNF is also disclosed.

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GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR RECEPTORS

Pursuant to 35 U.S.C. § 119(e), the present application claims priority
5 benefit of provisional application serial numbers 60/006,619, filed November 13, 1995; 60/015,767, filed April 16, 1996; 60/021,965, filed June 27, 1996; 60/020,638, filed June 27, 1996; and 20/020,639, filed June 27, 1996, all hereby incorporated by reference in their entireties.

10 FIELD OF THE INVENTION

The present invention relates to the identification of receptors for and functions of GDNF, and cell lines expressing the receptors.

BACKGROUND OF THE INVENTION

15 Glial cell line-derived neurotrophic factor (GDNF) is a trophic polypeptide. It is a disulfide bridge-linked homodimer of two 134-amino acids long glycosylated polypeptides, with a molecular weight of approximately 25-30 kD for each monomer. Prior to the molecular cloning of GDNF in 1993, investigators sought a trophic polypeptide which would alleviate the neuronal loss associated with
20 Parkinson's disease, specifically dopaminergic neurons of the ventral mesencephalon. The survival of this subpopulation of neurons has been known for some time to be promoted by soluble factors present in the conditioned media of glial cell lines. It was from one of these cell lines that the GDNF protein was initially isolated based upon its ability to promote dopamine uptake in primary
25 cultures prepared from embryonic ventral midbrain neurons (Lin et al., 260 Science 1120, 1993). Subsequently, GDNF was shown to promote survival of adult substantia nigra neurons *in vivo* following pharmacological treatments and lesions that mimic Parkinsonian syndromes (Beck et al., 377 Nature 339, 1995; Tomac et al., 373 Nature 335, 1995) Although GDNF was originally reported to be highly
30 specific for dopaminergic neurons, several other potent activities of this molecule have subsequently been demonstrated, including survival and phenotypic responses in facial and spinal motor neurons (Henderson et al., 266 Science 30 1062, 1994; Oppenheim et al., 373 Nature 344, 1995; Yan et al., 373 Nature 341, 1995).

noradrenergic neurons of the locus coeruleus (Arenas et al., *Neuron*, in press, 1995), cerebellar Purkinje cells (Mount et al., 92 PNAS 9092, 1995), sympathetic and sensory neurons in peripheral ganglia (Trupp et al., 130 J. Cell Biol. 137, 1995) and for populations of peripheral neurons with target-derived and paracrine mode of action (Trapp, M. et. al., *J. Cell Biol.*, 130, 137-148 (1995); Pitchel, J., Sariola, H., Hoffer, B. & Westphal, H. (unpublished observation); Buj-Bello, A., Buchman, V. L., Horton, A., Rosenthal, A. & Davies, A. M. *Neuron*, 15, 821-828 (1995). As many of these neurons are affected in neurodegenerative diseases, GDNF may have potent therapeutical applications. Particularly, exogenously administered GDNF maintains dopaminergic neurons of the substantia nigra in experimentally induced Parkinsons disease in rodents (Beck et al. (1995) *Nature*, 373, 339-341; Tomac et al. (1995) *Nature*, 373, 335-339) and leads to functional recovery in Parkinsonian rhesus monkeys (Gash et al. (1996) *Nature*, 380, 252-255). GDNF treatment also rescues about half of the experimentally axotomized murine motoneurons (Oppenheim et al. (1995) *Nature*, 373, 344-346; Li et al. (1995) *Proc. Natl. Acad. Sci. U.S.A.*, 92, 9771-9775) suggesting that GDNF may be used in treatment of motoneuronal diseases. The studies of the mechanism of GDNF action in normal and pathogenic conditions have been, however, basically hampered as its receptor was not known.

Based upon structural similarities (primarily seven conserved cysteine amino acid residues), GDNF appears to be a distant member of the transforming growth factor-beta (TGF- β) superfamily of multifunctional cytokines, which includes TGF- β s, activins, bone-morphogenetic proteins (BMPs) and growth and differentiation factors (GDFS) (Roberts et al., 327 *Philos.Trans.R.Soc.Land.* 145,1990). TGF- β and related ligands are known to suppress proliferation in epithelial and immune cells, to function as morphogens in early development, to induce ectopic expression of skeletal tissue, and to promote survival and differentiation of neurons. TGF- β superfamily proteins interact with numerous receptor subunits on the surface of responsive cells (Attisano et al., 1222 *Mol.Cell Res.* 71, 1994; Derynck, 19 *Trends Biochem. Sci.* 548, 1994). Different receptor types have been described based on the molecular weights of affinity labeled

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complexes. Among these are the type I, type II and type III receptors, which represent binding proteins of 55kD, 70kD and 300kD, respectively. Type III receptors are abundantly expressed transmembrane proteoglycans of approximately 300kD with a short cytoplasmic tail, and are thought to function in recruitment of ligand to an oligomeric receptor complex (Lopez-Casillas et al., 67 Cell 785, 1991). Indeed, a type III receptor is required on some cell lines for TGF- β 2 binding to the signaling receptors. Type I and type II receptors are transmembrane proteins with an intracellular serine-threonine kinase domain and can therefore transmit downstream signals upon ligand binding (Attisano et al., 75 Cell 671, 1993; Derynck, 1994 *supra*). Type II receptors are constitutively activated kinases which upon ligand binding recruit type I receptors to a signaling complex. In this complex, type I receptors are phosphorylated by type II receptors on a juxtamembrane domain rich in serine residues, this phosphorylation is thought to result in the activation of the ser-thr kinase activity of type I receptors and in downstream signaling (Wrana et al., 370 Nature 341, 1994). According to this model, TGF- β superfamily proteins can not bind to type I receptors in the absence of type II receptors, although in some cases, type I receptors are necessary for efficient binding to type II receptors (Letsou et al., 80 Cell 899, 1995). Multiple cDNA clones of type I, II and III receptors for TGF- β S, activins and BMPs have been isolated by either expression or homology cloning, including seven mammalian type I receptors, four type II receptors and one type III betaglycan receptor. Additional membrane proteins binding different members of this family include glycosylphosphatidyl inositol (GPS)-linked 150kD and 180kD proteins of unknown structure and function (MacKay and Danielpour, 266 J. Biol. Chem. 9907, 1991), and endoglin, a 180kD disulphide linked dimer which binds TGF- β 1 but not TGF- β 2.

The isolation and characterization of GDNF receptors is a prerequisite for the understanding of the full range of biological actions of GDNF and the signaling events that take place upon GDNF binding to responsive cells. Until now, progress in this area has been hampered by the lack of cell lines responsive to GDNF, that is, cell lines comprising GDNF receptors.

SUMMARY OF THE INVENTION

Receptors for GDNF are disclosed herein, as are cell lines expressing the same. Methods for identifying and isolating these receptors are also disclosed.

5 In one aspect, the present invention relates to isolated receptors which bind GDNF.

In another aspect, the present invention relates a method for determining compounds or compositions which bind GDNF receptors.

10 In yet another aspect, the present invention relates to methods for identifying homologs of GDNF by screening for compounds or compositions which have similar biological effects, such as tyrosine phosphorylation, increase in c-fos mRNA, and and increases in cell survival.

15 In still another aspect, the present invention relates to method for identifying analogs of GDNF by screening for compounds or compositions which are antagonistic for the biological effects of GDNF, such as are listed above.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1. Binding of ^{125}I -GDNF to receptors on chick sympathetic neurons. (a) Saturation steady-state binding of ^{125}I -GDNF to E10 embryonic chick sympathetic neurons. Data are expressed as mean \pm SD of triplicate determinations. (b) 20 Scatchard transformation of the data plotted in (a). (c) Hill transformation of the data plotted in (a) nH: Hill coefficient.

25 Fig.2. Affinity labeling of GDNF receptors on chick sympathetic neurons. ^{125}I -GDNF was cross-linked to E10 embryonic chick sympathetic neurons, receptor complexes were fractionated by SDS/PAGE and visualized by gel autoradiography (middle lane). A doublet at 100kD and a 300kD complex are indicated by arrows. Excess cold GDNF prevented cross-linking of ^{125}I -GDNF (right lane). For comparison, crosslinking of ^{125}I -TGF- β to mink lung epithelial cells Mv1Lu is also shown (left lane). Molecular weight markers are indicated in kD.

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Fig.3. Affinity labeling of GDNF receptors on cell lines. ^{125}I -GDNF was cross-

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linked

to C6 glioma, RN33B raphe nucleus, L6 myoblast and MN-1 motor neuron cell lines with either DSS or EDAC as crosslinker agents. Receptor complexes were fractionated by SDS/PAGE and visualized by gel autoradiography. Excess cold GDNF prevented cross-linking of ^{125}I -GDNF (cold). Molecular weight markers are indicated in kD.

Fig. 4. Individual constituent affinities of GDNF receptor subunits in RN33B and MN-1 cells. (a) Sizes of different GDNF receptor complexes on RN33B and MN-1 cells after cross linking with EDAC or DSS. (b) and (c) ^{125}I -GDNF was cross-linked to RN33B (b) or MN-1 (c) cells in the presence of increasing concentrations of cold GDNF. The percentage of ^{125}I -GDNF binding to the indicated receptor subunit is plotted as a function of the concentration of cold GDNF used during binding.

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Fig. 5. Expression of GDNF mRNA in cell lines expressing GDNF receptors. (a) Autoradiogram of an RNase protection assay using equal amounts of total RNA from the indicated cell lines. Kidney post natal day 1 and yeast tRNA were used as positive and negative controls, respectively. (b) Quantification of the level of GDNF mRNA in different cell lines relative to the level in PI kidney. undiff RN33B, undifferentiated RN33B cells; diff RN33B, differentiated RN33B cells; diff RN338+GDNF, RN33B cells differentiated in the presence of GDNF.

20

Fig. 6. Expression of mRNA for *c-ret* in different cell lines.

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Fig. 7. GDNF stimulation of tyrosine phosphorylation of ERKs in RN33B and MN-1 cells. RN33B (a) or MN-1 (b) cell monolayers were exposed to 50 ng/ml GDNF during the indicated periods of time (in minutes), cell lysates were fractionated by SDS/PAGE and Western blots probed with an anti-phosphotyrosine antibody (aP-Tyr). The blots were stripped and reprobed with an anti ERK2 antibody (a-ERK2) that recognizes both p42^{erk} 12 and p44^{erk} 12 (arrows to the right). Molecular weight markers are indicated in kD.

30

Fig. 8. GDNF stimulation of c-fos mRNA expression in RN33B and MN-1 cells. RN33B (a) or MN-1 (b) cell monolayers were exposed to 50 ng/ml GDNF during the indicated periods of times, total RNA was extracted and fractionated in agarose gels and Northern blots probed with a ³²P-labeled rat c-fos probe. Shown are x-ray autoradiograms of filters washed at high stringency.

Fig. 9. GDNF increased the survival of RN46A cells. RN46A cells were differentiated in media \pm 0-50 ng/ml GDNF for 8 days. The data represent the means \pm SEM of three independent experiments (1,500-3,000 cells counted per condition). ANOVA indicated that the GDNF had a significant effect on survival at all concentrations compared to media alone (overall ANOVA: df=6,203; F=1 1.39, p,0.001; unequal N LSD post hoc test, p,0.001).

Fig. 10 a-c. Biological and biochemical responses of MN-1 to GDNF. (a) GDNF stimulates survival of serum-deprived MN-1 cells. (b) GDNF stimulates rapid and transient tyrosine phosphorylation of several proteins (asterisks) in MN-1 cells. Time of GDNF treatment (in minutes), and molecular weight markers are indicated. (c) Rapid and sustained ERK1 and ERK2 tyrosine phosphorylation stimulated by GDNF in MN-1 cells.

Fig. 11 a-b. c-RET is a signal transducing receptor for GDNF. (a) Immunoprecipitation analysis of GDNF-receptor complexes in MN-1 cells. GDNF-labeled binding proteins could be precipitated with lectin Sepharose beads, or antibodies against GDNF, phospho-tyrosine (P-Tyr) and c-RET. Control preimmune antibodies did not immunoprecipitate GDNF receptor complexes. (b) GDNF induces tyrosine phosphorylation of c-RET in MN-1 cells. c-RET tyrosine phosphorylation was detected already 5 minutes after addition of GDNF (upper panel). Saturation was observed at 30 ng/ml GDNF (lower panel).

Fig. 12 a-b. *c-ret* expression is sufficient to mediate binding and biological responses to GDNF in fibroblasts. (a) Iodinated GDNF could be cross linked to 3T3 cells stably transfected with MEN2a-ret or wild type *c-ret* expression

plasmids. Untransfected 3T3 cells (3T3) did not bind GDNF. The specificity of the binding was demonstrated by displacement of the labeling with 50X excess cold GDNF. (b) GDNF promotes survival and growth responses in 3T3 fibroblasts stably transfected with a *c-ret* expression plasmid. Untransfected cells
5 did not respond to GDNF.

Fig. 13 a-c. *c-ret* mRNA expression in adult brain and in developing substantia nigra. (a) Ribonuclease protection analysis (RPA) of *c-ret* mRNA expression in different regions of the adult rat brain. (b) RPA of *c-ret* mRNA expression during
10 development of the rat ventral mesencephalon (nigra), and of GDNF mRNA expression in the developing striatum. (c) mRNA expression is indicated in arbitrary units where 100 corresponds to the level of expression in the respective regions in newborn animals.

15 Fig. 14 a-h. c-RET is expressed in GDNF-responsive substantia nigra dopaminergic neuron. (a) Dark field autoradiogram of *c-ret* mRNA expression analyzed by in situ hybridization in the adult substantia nigra. Scale bar, 40 μ m. (b) Bright field autoradiogram showing substantia nigra neurons containing *c-ret* mRNA. Scale bar, 7.5 μ m. (c) Immunohistochemical analysis of c-RET protein expression in the
20 adult substantia nigra. Scale bar, 27 μ m. (d) autoradiogram showing *in situ* hybridization for *c-ret* mRNA in the adult rat brain after a unilateral lesion with 6-OHDA. The injection of this toxic dopamine analogue in the medial forebrain bundle ensures that only cells which actively take up and retrogradely transport dopamine will be compromised. Note the disappearance of the labeling for *c-ret*
25 mRNA in the lesioned substantia nigra (arrowhead) 1 day and 5 days following the lesion. (e) Immunohistochemical analysis of c-RET protein expression in the adult substantia nigra after lesion with 6-OHDA and grafting of mock transfected fibroblasts (control graft). Note the nearly complete absence of c-RET-LI caused by the lesion. Scale bar, 20 μ m. (f) Grafting of GDNF-expressing fibroblasts
30 rescues c-RET-LI. Note c-RET positive fibers surrounding and entering the GDNF producing graft (arrows). Same magnification as in (e). (g) Immunohistochemical analysis of cRET protein expression in the adult locus coeruleus after lesion with

6-OHDA and grafting of mock transfected fibroblasts (control graft). Scale bar, 23 μ m. (h) Rescue of cell bodies expressing c-RET-LI by GDNF in of 6-OHDA lesioned locus coeruleus. Same magnification as in (g). Graft is on the right in (e) and (f), and above in (g) and (h).

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Fig. 15 a-c. PC12 and NB2/a cells respond to GDNF and bind GDNF. (a) GDNF promotes survival of serum-deprived PC12 cells. (b) GDNF increases the number of NB2/a cells. (c) 125 I-GDNF binds to PC12 and NB2/a cells in the absence (open column) or presence (filled column) of 50-fold unlabeled GDNF.

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Fig. 16. Affinity crosslinking of 125 I-GDNF to cell lines. 125 I-GDNF was crosslinked to PC12 cells (lane 1), SY5Y cells (lane 2), E20 rat kidney cells (lane 3) and NB2/a cells (lane 4), and the resulting complexes were precipitated from detergent lysates by anti-GDNF antibodies (Santa Cruz).

15

Fig. 17 a-b. GDNF specifically binds to c-RET. (a) 125 I-GDNF was crosslinked to NB2/a cells in the presence (+) or absence (-) of 1000-fold excess of unlabeled GDNF (PeproTech EC Ltd.), and the resulting complexes were precipitated from detergent lysates by cocktail of monoclonal and polyclonal (Santa Cruz) anti-c-RET antibodies recognizing the extracellular and intracellular domain of cRET, respectively. Lysates were also precipitated by monoclonal anti-neurofilament antibodies 13AA8 (lane 3), by Protein A-Sepharose (lane 4) and by WGA-Agarose (lane 5). (b) 125 I-GDNF binds to COS cells transiently expressing c-RET, but not to mock-transfected (with pBK-CNV plasmid) COS cells. Open column represents binding in the presence, and filled column in the absence of 50-fold excess of unlabeled GDNF.

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Fig. 18. GDNF increases tyrosine phosphorylation of c-RET in transfected COS cells. c-RET was immunoprecipitated from detergent lysates of GDNF-treated (+) (lane 1) or untreated (-) (lane 2) COS cells transfected (lane 3) with *c-ret* cDNA or mock-transfected with PBK-CMV plasmid. (a) immunoblot probed with anti-c-RET antibodies (Santa Cruz). (b) the same filter reprobed with anti-phosphotyrosine

30

antibodies.

Fig. 19 a-h. GDNF binds *in situ* to *c-ret*-positive developing enteric neurons. (a, b) Darkfield (a) and corresponding bright-field (b) microphotographs of GDNF antisense cRNA hybridization to paraffin sections through E15 rat gut. (c, d) Dark-field (c) and corresponding bright-field (d) microphotograph of *in situ* binding of ¹²⁵I-GDNF to E15 rat gut explants. (e) *c-ret* antisense cRNA hybridization to a cryosection through E15 rat gut. (f) Immunostaining of E15 rat gut cryosection with anti-peripherin antibodies. (g) GDNF sense cRNA hybridization to E15 rat gut section. (h) *In situ* binding of ¹²⁵I-GDNF to E15 rat gut explants in the presence of 250-fold excess of unlabeled GDNF. ---, muscle layer; n, intestinal nerve plexus. Bar, 100μm.

Fig. 20 a - b. Crosslinked GDNF-c-RET-complexes are obtained from GDNF-responsive cell lines and from *c-ret*-transfected cells (a) ¹²⁵I-GDNF was crosslinked with EDAC to PC12 cells, NB2/a cells, dissociated E20 rat kidney cells, and COS cells, and the resulting complexes were precipitated by anti-GDNF antibodies. (b) EDAC-crosslinked ¹²⁵I-GDNF-c-RET complexes were immunoprecipitated with anti-c-RET antibodies from the extracts of PC12 cells, stably *c-ret*-transfected (Ret.-3T3) or mock-transfected (mock-3T3) 3T3 cells, as well as from dissociated E15 kidney cells in the presence (+) or absence (-) of 500-fold excess of unlabeled GDNF or TGF-β1. The ~50K bands in all gels are the crosslinked dimers of GDNF.

Fig. 21 a - b. GDNF increases c-RET autophosphorylation in stably transfected 3T3 cell line. (a) GDNF dose-dependently increases tyrosine phosphorylation of 160 kD isoform of c-RET in *c-ret*-transfected (ret-3T3) but not in mock-transfected (mock) cells. (b) GDNF time-dependently increases tyrosine phosphorylation of 160 kD isoform of cRET in *c-ret*-transfected 3T3 cells. Upper panels (Ret.-PTyr) are the immunoblots stained with anti-phosphotyrosine antibodies, and lower panels (Ret.) show the reprobing of the corresponding filters with anti-c-RET antibodies.

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Fig. 22. GDNF increases the number of *trkC*-3T3 fibroblasts transiently expressing *c-RET* (open squares), but not mock-transfected cells (filled squares). *c-ret* and mock-transfected cells in five parallels were treated with rat GDNF at indicated concentrations, or with NT-3, for five days. Cell number, quantified with Abacus™ Cell Proliferation Kit (Clontech), is expressed as a percent of the control cells without growth factors. *, $p < 0.001$ compared to mock transfected cells.

Fig. 23 a-b. Purification of receptor from L6 myeloblast cells. (a) Plasmon resonance analysis of fractions obtained from anion exchange chromatography of L6 cell lysates. Total protein of fractions is also depicted. (b) Further purification of 1M fraction obtained from (a) by hydrophobic interaction chromatography.

Fig. 23. Autoradiographic film of the ligand blot ^{125}I -GDNF with proteins from adult rat brain (lane 2) and liver (lane 3). 50-fold excess of unlabeled GDNF (lane 1) significantly reduces the binding.

DETAILED DESCRIPTION

A prerequisite for the understanding of the full range and mechanisms of action of GDNF is the characterization of GDNF receptors and their signaling pathways. Although receptors for other members of the TGF- β superfamily are well characterized, GDNF receptors remained undefined until this disclosure. Disclosed herein is the biochemical characterization of GDNF receptors and their downstream responses in sympathetic neurons and responsive cell lines. Using affinity labeling, multiple GDNF binding subunits that mediate cooperative binding of GDNF to embryonic sympathetic neurons are identified. Screening of over thirty cell lines initially revealed high expression of GDNF binding proteins of 55 kD, 70 kD, 135 kD and 300 kD in conditionally immortalized neuronal precursors from the raphe nucleus. As the data demonstrate, GDNF receptors were highly induced after neuronal differentiation of these cells, which then became sensitive to the survival-promoting effects of GDNF. Different combinations of these subunits were also seen in glioma, myoblast and Sertoli cells. A different receptor pattern

was found in a motor neuron hybrid cell line, where the predominant component was a CPI-anchored protein of 155kD.

Despite the striking similarity with the receptor pattern of other TGF- β superfamily members, immunoprecipitation experiments indicated that GDNF receptor subunits of 55kD, 70kD, 135kD, and 300kD are novel proteins. The 155kD subunit was subsequently determined to be the product of the *c-ret* proto-oncogene, c-RET, a receptor tyrosine kinase crucial for the development of parts of the excretory and nervous systems. GDNF stimulated ERK tyrosine phosphorylation and *c-fos* mRNA expression with different time-courses in raphe nucleus and motor neuron cell lines, suggesting that different complements of GDNF receptor subunits can form distinct signaling complexes.

Concomitantly, c-RET was identified as receptor for GDNF on additional cell lines. GDNF rescues c-RET-positive dopaminergic and noradrenergic neurons in lesion models of Parkinson's disease, suggesting that cRET may mediate the anti-Parkinsonian effects of GDNF in the adult brain.

c-ret proto-oncogene (Takahashi et al. (1985) *Cell*, 42, 581-588) encodes a protein that is structurally related to receptor tyrosine kinases (Takahashi et al. (1988) *Oncogene*, 3,571-578). Its extracellular part contains an unusual cadherin-like domain and also a cysteine-rich domain, the biological roles for which are not understood. By alternative splicing, several isoforms of *c-ret* mRNA have been described (Tahira et al. (1990) *Oncogene*, 5, 97-102; Myers et al., (1995) *Oncogene*, 11, 2039-2045; Lorenzo et al. (1995) *Oncogene*, 10, 1377-1383), but their biological meaning is currently not understood. In several cell lines, *c-ret*-encoded proteins with molecular weights of 160 kD and 140 kD are described, representing the fully and partially glycosylated isoforms of 120 kD core protein, respectively (Takahashi et al., 1988). As with other receptor tyrosine kinases, c-RET is activated by homodimerization followed by phosphorylation of its tyrosine residues.

In the excretory system, *c-ret* is expressed in the nephric duct, the ureteric bud and the growing tips of the collecting ducts (Pachnis et al., (1993), *supra*). Mice homozygous for a null mutation in the *c-ret* gene die soon after birth, with kidneys either absent or rudimentary and displaying severe defects in the enteric

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nervous system (Schuchardt et al., *Nature* 367, 380-3 (1994). Based on this evidence, it had been proposed that the cognate *c-ret* ligand may be a growth factor important for morphogenesis and neurogenesis.

During murine embryogenesis, *c-ret* mRNA is expressed primarily in the nervous and excretory systems. *c-ret* mRNA is found in dorsal root, sympathetic, enteric and cranial ganglia (Pachnis et al., *Development* 119, 1005-17 (1993), as well as in post migratory neural crest cells and in various tumors of neural crest origin, including pheochromocytoma, medullary thyroid carcinoma and neuroblastoma (Ikeda, I., et al. *Oncogene* 5, 1291-6 (1990); Santoro, M., et al. *Oncogene* 5, 1595-1598 (1990). In the developing central nervous system, sites of *c-ret* expression include the ventral portion of the neural tube, the retina and motor neurons in spinal cord and hindbrain (Pachnis et al., (1993), *supra*). However, the pattern of expression of *c-ret* in the adult nervous system has not previously been reported.

The absence of a known ligand for c-RET has basically hampered the studies of intracellular pathways that c-RET can mediate. Comparative analysis of the growth-promoting activity of the epidermal growth factor receptor/c-RET chimera expressed in fibroblastic or hematopoietic cells revealed a biological phenotype clearly distinguishable from that of epidermal growth factor receptor (Santoro et al. (1994) *Mol. Cell. Biol.* 14, 663-675). We disclose herein that both NGF and GDNF promote survival of PC12 cells, whereas only NGF induces their differentiation, suggesting only a partial overlap in the signaling pathways of c-RET and trkA, a receptor for NGF. Binding of an adaptor protein Grb2 to oncogenic forms of c-RET has been demonstrated (Borrello et al. (1994) *Oncogene*, 9, 1661-1668). However, the details of the pathways are completely unknown. Now, having GDNF as a ligand, it is possible to address the intracellular signaling of c-RET upon GDNF binding.

Like c-RET, GDNF is abundantly expressed in the muscle layer of the gastrointestinal tract and in the condensing mesenchyme of the kidney (Suvanto et al. (1996) *Eur. J. Neurosci.*, 8, 816-822). Further, as disclosed herein, GDNF specifically binds c-RET-positive cells in developing gut, GDNF can be crosslinked to c-RET in several cell lines and in developing kidney, GDNF specifically induces

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tyrosine phosphorylation of c-RET, and ectopical expression of c-RET in 3T3 cells confers a biological response of these cells to GDNF. Thus, c-RET is activated by GDNF and mediates its functions.

5 The product of the *c-ret* proto-oncogene plays important roles in human disease. Rearrangements and mutations in the *c-ret* gene are associated with several tumors e.g. familial medullary thyroid carcinoma, multiple endocrine neoplasia type 2, etc., but also with Hirschsprung disease, a disorder that is characterized by the absence of enteric neurons in the hindgut, resulting in obstipation and megacolon in infants and adults (reviewed in Mak, Y. F. and
10 Ponder, B.A. J. (1996)*Curr. Op. Genet. Dev.*, 6, 82-86). Identification of GDNF as a ligand for c-RET further enables the analysis of the molecular basis of these diseases. Particularly, the mutations in GDNF gene can now be studied as possible cause for the Hirschsprung disease in the cases where *c-ret* locus is not mutated.

15 The phrases "GDNF receptor" and "receptor for GDNF" as used herein each refer to a single subunit which binds GDNF as well as combinations of the receptor subunits which bind GDNF.

The term "effect" as used herein means an alteration or change. An effect can be positive, such as causing an increase in some material, or negative, e.g., antagonistic or inhibiting.

20 The term "homolog" as used herein refers to a compound or composition having a similar biological effects as GDNF, such as are disclosed herein.

The term "analog" as used herein refers to a compound or composition having an antagonistic effect on the biological effects of GDNF.

25 The term "isolated" as used herein in reference to a GDNF receptor means a compound which has been separated from its native environment or, if recombinantly expressed, from its expression environment.

30 The phrase "substantially pure" as used herein in reference to a compound means an isolated compound which has been separated from other components which naturally accompany it. Typically, a compound is substantially pure when it is at least 75%, more preferably at least 90%, and most preferably 99% of the total material as measured, for example, by volume, by wet or dry weight, or by mole percent or mole fraction.

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The phrase "non-permissive culture conditions" as used herein refers to conditions which do not normally support survival of the cells being cultured *in vitro*, e.g., temperature, media components, etc.

5 The phrase "an excess" as used in reference to the addition of labeled GDNF in a competitive assay refers to an amount of labeled GDNF sufficient to facilitate the detection of a competing compound -- for example, an amount of labeled GDNF which is twice the amount of the compound to be tested.

10 The term "bind" as used herein refers to the interaction between the GDNF ligand and its receptor, the binding being of a sufficient strength and for a sufficient time to allow the detection of said binding under the conditions of the assays disclosed herein.

The term "about" in reference to a numerical value means $\pm 10\%$ of the numerical value, more preferably $\pm 5\%$, most preferably $\pm 2\%$.

15 The specific examples presented below demonstrate that:

- 1) GDNF receptor is present in multiple neuronal and non-neuronal cell types;
- 2) GDNF receptor is composed of multiple subunits which cooperate to achieve high affinity binding;
- 20 3) members of the ERK/MAP kinase family are components of the GDNF signaling mechanism; and
- 4) c-RET is a functional receptor for GDNF.

25 **1. Novel GDNF receptor expression in multiple neuronal and non-neuronal cell types**

Heretofore unidentified receptors are identified herein as GDNF receptors. These novel GDNF receptors were found in cells lines of different origins, although they appeared to be most abundant in neuronal cells. Preferably, the cell lines are selected from the group consisting of RN33B, RN46A, and C6 (see Table 1), with RN33B being most preferred. The identification of GDNF receptors in
30 many of these cell types suggests novel cellular populations responsive to GDNF *in vivo*. GDNF has been shown to promote survival and phenotype of distinct

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subpopulations of neurons, in particular dopaminergic and noradrenergic central neurons, as well as spinal and facial motor neurons. Given the activities of GDNF in various monoaminergic neurons, the discovery of GDNF receptors in cell lines derived from the medullary raphe indicate that serotonergic neurons may also respond to GDNF *in vivo*. The endogenous expression of GDNF by these cells suggests that this factor may act in a paracrine/autocrine fashion within the raphe nucleus. Expression of GDNF receptors in Sertoli TM4 cells suggests non-neuronal roles for GDNF in developing testis. *In vivo*, the temporal expression of GDNF mRNA in testis correlates with the expansion of the Sertoli cell population (Trupp et al., *supra*) which, together with the discovery of GDNF receptors on the TM4 cell line, suggest an autocrine action of GDNF during Sertoli cell maturation. Similarly, the presence of GDNF receptors in rat myoblast L6 cells, together with the expression of in developing muscle *in vivo* (Henderson et al., *supra*; Trupp et al., *supra*), indicates a potential paracrine role of GDNF during myogenesis.

Despite the presence of receptors and biological activities of GDNF on embryonic sympathetic neurons, PC12 cells which had been differentiated into sympathetic-like neurons with NGF did not express GDNF receptors under initial experimental conditions. As discussed below, however, GDNF receptors were ultimately identified on PC12 cells.

GDNF receptors are absent in the pons noradrenergic cell line CATH.a. Given the robust effects of GDNF on adult central noradrenergic neurons from the locus coeruleus, the absence of GDNF receptors in CATH.a is intriguing. Recently, however, Gong et al. reported that GDNF can prevent the degeneration of CATH.a cells induced by 6-OH-dopamine treatment (Gong et al., 21 *Abs. Soc. Neurosci.*, 1789, 1995), suggesting that GDNF receptors may be induced in these cells after 6-OH-dopamine lesion. Indeed, *in vivo* studies have shown that GDNF elicits a more profound induction of the phenotype of noradrenergic neurons following 6-OH-dopamine injection than in the non-lesioned locus coeruleus. Further, Treanor et al. recently reported upregulation of GDNF binding in sections of the substantia nigra after medial forebrain bundle transaction (Treanor et al., 21 *Abs. Soc. Neurosci.* 1301, 1995), suggesting that the receptor upregulation may be a general mechanism of control of GDNF responsiveness in the central nervous

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system.

GDNF receptor upregulation was also observed during *in vitro* differentiation of raphe nucleus cells. These lines have recently been shown to retain the ability to respond to local microenvironmental signals after
5 transplantation into the adult brain, where they differentiate in a direction that is consistent with that of endogenous neurons in the transplantation site (Shihabuddin et al., 15 *J. Neurosci.* 6666, 1995). *In vitro*, however, a shift to the non-permissive temperature differentiates them along default pathways into glutamatergic (RN33B) or serotonergic (RN46A) phenotypes, respectively.
10 Differentiation in culture has also been shown to upregulate expression of receptors for other trophic factors in these cells, including the neurotrophin receptors p75^{LNGFR} and trkB (Whittemore and White, 615 *British Res.* 27, 1993). Although they can give rise to different neuronal types depending upon the site of transplantation, RN33B cells are not able to generate glial elements, suggesting
15 these cells represent neuronally restricted multipotent precursors (Shihabuddin et al., *supra*). In this respect, it is interesting to note the absence of GDNF receptors in two pluripotent neuronal stem cell types (Renfranz et al., 66 *Cell* 713, 1991; Snyder et al., 30 68 *Cell* 33, 1992) suggesting that these cells are less restricted than the raphe nucleus cell lines. Taken together, these observations suggest that
20 GDNF receptor expression may initially appear in newly differentiated post-mitotic neurons and increase progressively during neuronal maturation.

2. Multiple GDNF receptor subunits

The data demonstrate that novel GDNF receptor is composed of multiple
25 subunits which cooperate to achieve high affinity binding. The cooperative binding of GDNF to embryonic sympathetic neurons may thus be an indication of a multi-step mechanism of receptor assembly. Because binding assays were performed at 4°C, binding cooperativity is unlikely to have resulted from substantial lateral mobility of transmembrane receptor proteins, suggesting that GDNF binding
30 induces conformational changes on receptor complexes that are partially preformed on the membrane. The nearly identical affinities of the different GDNF receptor subunits obtained by crosslinking also support the notion of cooperative binding of

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GDNF to a partially pre-assembled receptor complex.

The structural similarities between GDNF and members of the TGF- β superfamily suggest that receptors for GDNF might conform to some of the prototypes described for receptors of members of the TGF- β family. Indeed, the pattern of GDNF binding proteins described herein is strongly reminiscent of type I, type II and type III TGF- β receptors.

Despite the overall similarities between GDNF and TGF- β superfamily receptors, no GDNF receptors could be detected in several cell lines known to express various TGF- β and activin receptor subunits, including the mink lung epithelial cell line Mv1Lu. In agreement with this observation, no binding of GDNF has been detected in COS cells transfected with different combinations of known type I and type II TGF- β superfamily receptors (Ibanez, C., unpublished; P. ten Dijke, personal communication), including the recently isolated type II receptor for BMPs (Rosenzweig et al., 92 *PNAS USA* 7632, 1995) and a novel brain-specific type I receptor (Ryden et al., 21 *Abs. Soc. Neurosci* 1754, 1995). Moreover, no GDNF receptor complexes could be recovered after immunoprecipitation with antipeptide antisera against any of the cloned TGF- β superfamily receptors, indicating that GDNF receptor components are novel proteins.

20

3. *c-RET* is a receptor for GDNF

GDNF receptors were found in a motor neuron-neuroblastoma hybrid cell line, but not in a basal forebrain cell which was also a hybrid with the same neuroblastoma, suggesting that the receptors detected on MN-1 cells represent physiologically relevant motor neuron GDNF receptors. In contrast to raphe nucleus cells, GDNF expression could not be detected in the motor neuron cell line, consistent with a target-derived mode of action for muscle-derived GDNF *in vivo* (Henderson et al., 266 *Science* 1062, 1994; Trupp et al., *supra*). GDNF binds to and induces tyrosine phosphorylation of these receptors which were identified as the product of *c-ret*. *c-ret* was also able to mediate GDNF binding and survival/growth responses to GDNF upon transfection into naive fibroblasts. Moreover, dopaminergic neurons of the adult substantia nigra were found to

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express high levels of *c-ret* mRNA, and c-RET expressing dopaminergic and noradrenergic neurons in the CNS responded to the protective effects of exogenous GDNF *in vivo*. Together, these data indicate that the product of the *c-ret* proto-oncogene encodes a functional receptor for GDNF which may mediate the neurotrophic effects of this factor on dopaminergic, noradrenergic and motor neurons.

The results disclosed herein indicate that the c-RET receptor tyrosine kinase is a signal transducing receptor for GDNF. This finding is surprising, given that all receptors for members of the TGF- β superfamily characterized so far are receptor serine-threonine kinases (Derynck, R. *Trends Biochem Sci* 19, 548-553 (1994); Attisano et al., *J.Bba-Mol Cell Res*, 222, 71-80 (1994)). GDNF is in fact a very divergent member of the TGF- β superfamily, with which it shares primarily the spacing between conserved cysteine residues in the amino acid sequencer. Its ability to interact with a receptor tyrosine kinase indicates a further functional divergence from other members of the TGF- β superfamily. Conversely, these findings could suggest that other TGF- β superfamily members may also utilize receptor tyrosine kinases.

The following results disclosed herein also implicate the *c-ret* proto-oncogene product as a functional receptor for GDNF:

- i) GDNF binds to COS cells ectopically expressing the *c-ret* proto-oncogene;
 - ii) GDNF can be chemically crosslinked to the product of the *c-ret* proto-oncogene ectopically expressed in COS cells
- or
- from NB2/a and PC12 cells;
 - iii) the *c-ret* proto-oncogene product ectopically expressed in COS cells, but also in NB2/a cells, becomes rapidly phosphorylated
 - iv) GDNF promotes biological effects i.e. mitogenic or trophic in cells expressing *c-ret* proto-oncogenic products.

GDNF specifically binds to RET-expressing (Figure 19 c, d, h) enteric neurons and the tips of ureteric buds in developing kidney. These tissues were absent or severely reduced in *c-ret*-deficient mice (Schuchardt et al. (1994) *Nature*, 367,380-383; Durbec et al. (1996) *Development*, 122, 349-358). The data disclosed herein further demonstrate GDNF-c-RET complexes from GDNF-responsive and *c-ret*-transfected cells and from embryonic kidney cells. Finally, GDNF time and dose-dependently activates c-RET, and introduction of *c-ret* into GDNF-nonresponsive cells results in GDNF-responsiveness.

10 **4. Downstream signaling pathways activated by GDNF receptor**

Investigation of GDNF signal transducing mechanisms in raphe nucleus and motor neuron cell lines has been conducted. The downstream responses elicited by GDNF in these cells demonstrate that the GDNF binding proteins identified herein represent functional GDNF receptors. The initial biochemical characterization of GDNF signal transduction pathways has identified members of the ERK/MAP kinase family as components of the GDNF signaling mechanism. ERK/MAP kinase activation by phosphorylation is the final step in a cascade of kinases that is set in motion after activation of the Ras pathway by various growth factors, including TGF- β (Yan et al., 269 *J. Biol. Chem.* 13231, 1994; Hartsough and Mulder, 270 *J. Biol. Chem.* 7117, 1995) and nerve growth factor (Thomas et al., 68 *Cell* 1031, 1992; Wood et al., 68 *Cell* 10 II, 1992). More recently, ERK2 has also been shown to form part of the signal transduction pathway activated by several cytokines, such as interferons and interleukins, which are not known to activate Ras (David et al., 269 *Science* 1721, 1995). Whether or not Ras activation is one of the steps in the signaling transduction mechanism of GDNF is an area of further interest.

Interesting differences were found between the patterns of ERK phosphorylation induced by GDNF in raphe nucleus RN33B cells and in motor neuron MN-1 cells. GDNF treatment stimulated very rapid (maximum at 5 min) and transient (undetectable after 60 min) tyrosine phosphorylation of ERK1 and ERK2 in RN33B cells, but relatively slower (maximum at 15 min) and more sustained (still detectable after 120 min) phosphorylation of ERK2, but not ERK1.

in MN-1 cells. That these differences may have functional significance is suggested by recent observations made in PC12 cells treated with different growth factors. Exposure of PC12 cells to NGF or fibroblast growth factor (FGF) results in neuronal differentiation and in sustained elevation of Ras activity and ERK tyrosine phosphorylation (Qiu and Green, 7 *Neuron* 977, 1991). In contrast, treatment with epidermal growth factor, which stimulates DNA synthesis and proliferation of PC12 cells, results in only transient (< 1 hr) activation of Ras and ERKs (Qiu and Green, 1991). Thus, different time-courses of ERK activation underlie different biological responses in PC12 cells. Taken together, the different patterns of GDNF receptors and GDNF-induced ERK phosphorylation in RN33B and MN-1 cells suggest that different GDNF receptor subunits can cooperate to assemble distinct signaling complexes in different cell types. Whether different GDNF signal transduction pathways underlie the different biological effects of GDNF is an area of further interest.

Upon activation, ERKs translocate to the nucleus where they phosphorylate and thereby regulate the activity of transcription factors which, in turn, control gene expression. Phosphorylation of p67^{SRF} and p62^{TCF} transcription factors recruits them to the serum response element (SRE) in the c-fos gene promoter and stimulates c-fos gene transcription (Gille et al., 358 *Nature* 414, 1992). Transcription of c-fos is rapidly and transiently induced after various stimuli, including exposure of PC12 cells to NGF (Millbrandt, 83 *PNAS USA* 4789, 1986) and of osteoblastic cells to TGF- β (Machwate et al., 9 *Mol. Endocrin.* 187, 1995).

c-fos forms part of the AP-1 transcription factor complex, which is thought to be involved in the regulation of multiple genes, including growth factor, neuropeptide and neurotransmitter synthesizing enzyme genes (Gizang-Ginsberg and Ziff, 4 *Genes Dev.* 477, 1990); Hengeler et al., 87 *PNAS USA* 3899, 1990; Jalava and Mai, 9 *Oncogene* 2369, 1994). The stimulation of c-fos transcription by GDNF indicates a role for AP-1 complexes in GDNF-induced gene expression. Thus, c-fos could mediate the increase in the tyrosine hydroxylase (TH) expression observed upon GDNF treatment of central noradrenergic neurons, or the GDNF-induced upregulation of vasoactive intestinal peptide (VIP) and preprotachykinin-A (PPTA) mRNAs in cultured sympathetic neurons from the superior cervical

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ganglion (Trupp et al., 130 J. Cell Biol. 137, 1995).

The effects of GDNF on the survival of differentiated serotonergic raphe nucleus cells indicate that the GDNF receptors identified on these cells are able to elicit relevant biological responses. The fact that cessation of proliferation, differentiation, and GDNF responsiveness were concomitant with increased GDNF receptor expression in these cells, suggests that GDNF may be a survival factor for developing serotonergic raphe neurons *in vivo*. The data of this patent disclosure suggest a role for ERKs and c-fos in GDNF-mediated neuron survival. This can be directly established using dominant negatives or antisense oligonucleotides.

The GDNF receptor subunits and complexes disclosed herein have wide-range applicability. The identification and isolation of GDNF receptor facilitates rational drug design for drugs useful in treating, for example, neuronal disorders, particularly those involving neuronal cell death. As was discussed previously, GDNF has been shown to promote survival of adult substantia nigra neurons *in vivo* following pharmacological treatments and lesions that mimic Parkinsonian syndromes, as well as survival responses in other neuronal cell lines. The drugs can be tested for binding affinity to gdnf receptor, and for their influence on the downstream effect of GDNF disclosed below -- i.e., the phosphorylation of ERK2 and ERK1. As GDNF receptor has also been identified on malignant cell lines, design of drugs for use in cancer therapy is also evident. Further, considering structural similarity with BMP, the development of drugs to be used in treating bone-related diseases, i.e., osteoporosis, and for promoting the healing of fractures is also contemplated.

Accordingly, isolated receptors according to the present invention can be used, *inter alia*, to screen for compounds or compositions which are analogs and homologs of GDNF. The potential analogs and homologs can be screened initially in competitive binding assays employing either isolated receptor or cell lines expressing the receptor -- i.e., NB2/a cells -- and ¹²⁵I-labeled GDNF. Methods such as those disclosed in Example 13 can be used. Analog or homolog activity can then be ascertained by further identifying those compounds or compositions which, for example, effect a decrease or increase, respectively, in the tyrosine phosphorylation of the RET proto-oncogene. Methods such as those disclosed in

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Example 17 can be used. Alternatively, GDNF can be used to screen for and identify other receptors using the above-reference procedures, or variations thereof.

5 The isolation of GDNF receptor also facilitates the development of antibodies, both polyclonal and monoclonal, against the receptor. These antibodies can be used to purify the receptors themselves, identify other cells expressing GDNF receptor, thereby prompting other therapeutic applications, identify other Type I-interactive receptors, as well as be used as drugs themselves. The antibodies can initially be produced using the ligand/receptor complexes disclosed herein as the immunogens. Antibodies specific for the ligand can be eliminated
10 from the polyclonal serum by absorption with the ligand. Hybridomas for monoclonal production can be selected on the basis of binding of ligand, with the expansion of only those clones which do not bind the ligand uncomplexed with the receptor. The antibodies can be prepared by methods well known to those skilled in the art.

15 Alternatively, monoclonal and polyclonal antibodies against GDNF receptor and GDNF proteins can be used for the characterization and/or isolation of GDNF receptor molecular clones. Further, anti-GDNF antibodies can potentially be used in a screen for homologs, or in the production of anti-idiotypic antibodies which mimic GDNF.

20 The isolation of GDNF receptor also facilitates the isolation and/or production of nucleic acids for the expression of recombinant GDNF receptor, both *in vitro* and *in vivo*, for diagnostic and therapeutic applications. The term "nucleic acids" as used herein includes, for example, genomic DNA, mRNA, and cDNA. Upon
25 sequencing at least a portion of the GDNF receptor, oligonucleotide primers for isolating genomic DNA for GDNF receptor and receptor mRNA can be developed.

cDNA can be prepared from isolated mRNA. The isolation and
30 production of nucleic acids can be accomplished utilizing methods well known to those skilled in the art using standard molecular biology techniques such as are set forth in Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring

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Harbor Laboratory, Cold Spring Harbor, New York, 1982, incorporated herein by reference. Recombinantly produced receptors can be used in crystallography studies for rational drug design. Recombinant extracellular domain can be produced and used as a drug in ligand sink applications, e.g., for ligands with antagonistic properties.

The nucleic acids as set forth above can be utilized for gene therapy, using both *in vivo* and *ex vivo* techniques. The nucleic acids can also be used to clone other related receptors using, for example, low stringency screens and reversed transcriptase PCR; and to produce cells overexpressing the receptors to screen for other ligands, e.g., by panning, and other materials serving as receptor agonists, antagonists, or partial agonists and antagonists. Alternatively, recombinantly produced receptor itself can be used for the screening assays. Additionally, cells expressing chimeric receptors can be produced using other TGF- β receptor family members to elucidate signal pathways. Intracellular targets of GDNF receptor can be identified using, for example, the yeast two-hybrid system. (Chen, et al., 377 *Nature* 548, 1995, incorporated herein by reference.)

The nucleic acids set forth above can also be used to develop transgenic and/or gene targeted animals. For example, transgenic animals can be developed for testing the effects of the overexpression of GDNF receptor. Procedures can be utilized such as are described in Hogan et al., Manipulating the Mouse Embryo: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1986, and Capecchi, M.R., *Trends Genet*, 5: 70-76, 1989, both incorporated herein by reference.

Alternatively, cell lines and transgenic animals unable to express GDNF receptor can be prepared to ascertain the effects of blocking signaling by GDNF. Procedures such as are set forth in Wurst et al., *Gene Targeting* Vol. 126, edited by A. L. Joyner, IRL Press, Oxford University Press, Oxford, England, pp. 33-61, 1993, incorporated herein by reference, can be utilized.

Other applications and modifications are within the spirit and scope of the invention as herein disclosed and will be readily apparent to those skilled in the art.

EXAMPLES

The following Examples are provided for purposes of elucidation and not limitation on the disclosure or claims.

Unless otherwise indicated, binding and biochemical studies were carried out with recombinant rat GDNF produced in Sf21 insect cells using a baculovirus expression system. The protein was produced and purified as previously described (Trupp et al., *supra*, incorporated herein by reference). GDNF protein was quantified after silver staining of SDS/PAGE gels using standard curves obtained with commercial samples of proteins of molecular weight similar to that of GDNF. Purified human TGF- β 1 was generously provided by Jun-ichi Koumegawa, Kirin Brewery, Tokyo, Japan. Proteins were labeled with Na- 125 I by the chloramine-T method to a specific activity of approximately 1×10^8 cpm/ μ g.

Unless otherwise indicated, binding assays were performed as follows. Cells were incubated with iodinated GDNF in Dulbecco's phosphatebuffered saline and 2 mg/ml bovine serum albumin (BSA) on Millipore Hydrophilic Durapore 96-well filtration plates. Following two hours of vigorous shaking at 4°C, the cells were washed twice with ice-cold binding buffer under vacuum. Dried filters were liberated and bound 125 I-GDNF quantified in a gamma counter. Non-specific binding was determined by addition of 500-fold excess of cold ligand to the binding mixtures.

For affinity labeling, iodinated proteins were bound to monolayer cultures of primary neurons or cell lines. Prior to binding, dissociated chick sympathetic neurons were cultured for 48 hours in the presence of NGF on polyornithine/laminin coated dishes. Plated cells were incubated with 10 ng/ml 125 I-GDNF at 4°C in binding buffer as described above. Ligand/receptor complexes were chemically cross-linked for thirty minutes at room temperature using either disuccinimidyl suberate (DSS) or 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDAC) (Pierce Chemical, Rockland, IL). Following quenching of the cross-linking reactions, cells were washed twice with 10mM Tris/HCl buffered saline, 2 mM EDTA, 10% glycerol, 1% NP-40, 1% Triton x-100, 10 μ g/ml leupeptin, 10 μ g/ml antipain, 50 μ g/ml aprotinin, 100 μ g/ml benzamidine hydrochloride, 10 μ g/ml pepstatin and 1 mM PMSF (proteinase inhibitors from Sigma). Cleared lysates were boiled for 5 min in SDS/ β -mercaptoethanol buffer,

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fractionated by SDS/PAGE on 4-20% gradient electrophoresis gels, and visualized by autoradiography. Molecular weights indicated were obtained by subtracting the weight of a GDNF ligand monomer, e.g., 25-30 kD, more preferably 23 kD, from the estimated molecular weights of cross-linked complexes visualized by SDS/PAGE. For affinity measurements of cross-linked complexes, cells were incubated on plates as above in the presence of increasing amounts of unlabeled GDNF. These samples were fractionated by gradient SDS/PAGE, gels were then dried and specific bands excised according to molecular weights determined from autoradiograms, and count in a gamma counter. For immunoprecipitation of affinity labeled receptor complexes, after binding and cross-linking with iodinated ligands, cell lysates were cleared and incubated overnight at 4°C with 5-10 µl of anti-peptide rabbit antisera against difference type I, II and III TGF-β superfamily receptors (ten Dijke et al., 264 Science 101, 1994) (provided by Peter ten Dijke, Ludwig Institute for Cancer Research, Uppsala, Sweden). Immunocomplexes were collected with Protein A-Sepharose (Pharmacia, Sweden), washed in lysis buffer and boiled for 5 minutes before SDS/PAGE and autoradiography as above.

Example 1

GDNF receptors on embryonic sympathetic neurons

GDNF promotes survival of cultured embryonic chicken sympathetic neurons with similar efficacy and dose response curve as nerve growth factor (NGF) (Trupp et al., *supra*). Chicken sympathetic neurons, isolated and prepared as previously described (Trupp et al., *supra*). Saturation binding with iodinated GDNF was carried out on neurons isolated from embryonic day 10 (E10) chick paravertebral sympathetic ganglia mechanically dissociated in the presence of trypsin. The preparation was preplated for two hours on untreated tissue culture plastic in order to enrich in neurons and allow for re expression of receptors. Plots of saturation binding data produced a sigmoidal curve from which a K_d of 400 pM could be approximated (Fig. 1a). In agreement with the sigmoidal behavior of this curve, Scatchard transformation of the data produced an inverted U-shaped curve indicative of cooperative binding (Fig. 1b). The measure of cooperativity of

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binding can be ascertained from a Hill transformation which produced a positive slope of 1.63 (Fig. 1c), suggesting oligomerization of either ligand or receptor subunits.

In order to identify GDNF binding components on the membrane of sympathetic neurons, chemical cross-linking of ^{125}I -GDNF to these cells was utilized, followed by visualization of the resulting complexes by SDS-PAGE. Gradient gel electrophoresis resolved binding proteins of 70 and 300kD (molecular weights of GDNF receptor subunits reported hereafter were obtained by subtracting the weight of a GDNF ligand monomer, e.g., 23kD, from the estimated molecular weights of cross-linked complexes visualized by SDS/PAGE), resulting in a pattern of bands which resembled that obtained after cross-linking of TGF- β 1 to Mv1Lu mink lung epithelial cells (Fig.2). This result suggested that, like TGF- β receptors, GDNF binding proteins may also form an oligomeric receptor system. A large excess of cold ligand displaced iodinated GDNF from the receptor complex indicating the specificity of the labeling.

Example 2

GDNF receptors on cell lines

Over thirty cell lines were screened for expression of GDNF receptors using affinity labeling with iodinated GDNF (Table I, *infra*). Except as otherwise noted, all cell lines used in this study are available from and described by the American Type Culture Collection, Rockville, MD. A875 human neuroblastoma was provided by Mart Saarma, University of Helsinki, Finland. CATH.A, a noradrenergic cell line isolated from a tumor in the pons of transgenic mice expressing SV40 T antigen under the transcriptional control of a tyrosine hydroxylase promoter (Suri et al., 1993), was generated and provided by Dona Chikaraishi, Tufts University School of Medicine, Boston, MA. The rat neural stem cell line C17-2 (Snyder et al., 68 Cell 33, 1992) was generated and provided by Evan Snyder, Harvard Medical School, Boston, MA. LAN5 human neuroblastoma was provided by Sven Pahlman, Uppsala University, Sweden. David Hammond, University of Chicago, produced and provided SN6 cells, a hybrid of mouse basal forebrain cholinergic neurons and the mouse neuroblastoma N18TG2

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(Hammond et al., 1986). Human neuroblastoma SY5Y was provided by David Kaplan, ABL-Basic Research Program, NCI-Frederick Cancer Research and Development Center, Frederick, MD. ST15A rat neural stem cell line was kindly provided by Ron McKay, National Institute of Health, MD. The generation and characterization of raphe nucleus cell lines RN33B and RN46A has been described elsewhere (Whittemore and White, 1993). RN33B and RN46A cells were obtained from Dr. Scott Whittemore of the University of Miami. The motor neuron hybrid cell line 2FI.10.14 (referred to here as MN-1) has been previously described (Salazar-Grueso et al., 2 *Neuroreport* 505, 1991).

Multiple GDNF receptor subunits were detected in various glial, neuronal and non-neuronal cells (Table I). A large molecular weight band of 300kD appeared to be the most prevalent species in several cell lines after crosslinking with disuccinimidyl suberate (DSS), and it was the only receptor which appeared to bind ligand in the absence of all other receptors (Table I). A similar pattern was seen in rat C6 glioma, mouse Sertoli TM4 cells, and in two cell lines derived from embryonic neuronal precursors of the rat raphe nucleus, which have previously been shown to express multiple neuronal markers, including glutamate- (RN33B) and serotonin- (RN46A) synthesizing enzymes (Whittemore and White, 615 *Brain Res.* 27, 1993; White et al., 14 *J. Neuro.*, 1994; Eaton et al., 170 *Dev. Biol.* 169, 1995). The consensus pattern in these cells after cross-linking with DSS consisted of the large molecular weight band of 300kD, and two other receptor subunits with molecular weights at 50-55kD and 65-70kD, respectively (Fig. 3 and Table I). The 50-55kD component often ran as a doublet or triplet. The smeary appearance and heterogeneous range of sizes displayed by the large molecular weight component suggests a post-translational modification, presumably glycosylation, and appears similar to that previously described for type III betaglycan TGF- β receptors. This species was somewhat smaller in the cells derived from the raphe nucleus, which could indicate either a distinct core protein or difference levels of glycosylation.

GDNF receptors could not be detected in pheochromocytoma PC12 cells under the present assay conditions of 4°C, even after NGF-induced differentiation into a sympathetic neuron-like phenotype (Table I, and data not shown). No or

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very low GDNF receptor expression could be seen in various neuroblastomas, and in two pluripotent neuronal stem cells (Table I).

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TABLE I

	CELL LINE	DESCRIPTION	55kD	70kD	135kD	155kD	300kD
5	A875	human melanoma	-	-	-	-	+
	Balb.SFME	mouse embryonic cell	-	-	-	-	-
	CATH.a	rat pons noradrenergic	-	-	-	-	-
	Mv1Lu	mink lung epithelia cell	-	-	-	-	-
	COS-7	monkey kidney fibroblast	-	-	-	-	-
10	C2-C12	mouse myoblast	-	-	-	-	-
	C6	rat glioma	+	+	+	-	+
	C17-2	rat CNS stem cell	-	-	-	-	-
	FR-3T3	rat fibroblast	-	-	-	-	-
	HELA	human cervical carcinoma	-	-	-	-	-
15	LAN5	human neuroblastoma	-	-	-	-	+
	L6	rat myoblast	-	-	+	-	+
	MN-1	mouse motor neuron	-	+	-	+	+
	NB41A3	TH+ mouse neuroblastoma	-	-	-	-	-
	NRK-49F	rat kidney fibroblast	-	-	-	-	-
20	PC12	rat pheochromocytoma	-	-	-	-	-
	P19	mouse embryo carcinoma	-	-	-	-	-
	RN33B	rat raphe nucleus (glutamat)	+	+	+	-	+
	RN46A	rat raphe nucleus (seroton)	+	+	+	-	+
	SK-N-MC	human neuroepithelioma	-	-	-	-	-
25	SK-N-SH	DRH+ mouse neuroblastoma	-	+	-	-	+
	SN6	mouse basal forebrain (cholin)	-	-	-	-	-
	ST15A	rat CNS stem cell	-	-	-	-	+
	SW1353	human chondrosarcoma	-	-	-	-	-
	SY5Y	human neuroblastoma	-	-	-	-	-
30	TM3	mouse Leydig cell	-	-	-	-	-
	TM4	mouse Sertoli cell	+	+	+	-	+
	U138MG	human glioblastoma	-	-	-	-	-

35 Presence (+) or absence (-) of specific GDNF receptor complexes in the designated cell line.

Affinity labeling using the cross-linker ethyl-dimethyl-aminopropyl carbodiimide (EDAC) revealed the presence of an additional GDNF receptor component of 120-135kD (Fig. 3), only seen after very long exposure of gels in DSS cross-linked complexes. Like DSS, EDAC also cross-linked GDNF to

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receptors of 50-55kD and 65-70kD; the high molecular weight subunit of 300kD was, however, not as efficiently cross-linked by EDAC (Fig.3).

The raphe nucleus cell lines are only conditionally immortalized and do not show signs of transformation. At the non-permissive temperature and in defined medium, they stop proliferating and differentiate into postmitotic neurons (Whittemore and White, 1993). GDNF binding was greatly increased in RN33B and RN46A cells following differentiation (not shown). The overall pattern and the relative amounts of GDNF receptor components did not change after differentiation.

Analysis of GDNF binding proteins on the rat myoblast cell line L6 revealed a different pattern of receptor subunits marked by the apparent absence of 50-55kD and 65-70kD receptors. Only the high molecular weight component of 200-400kD could be seen after cross-linking with DSS (Fig. 3). Crosslinking with EDAC, however, readily labeled the 120-135kD subunit previously seen in C6. TM4 and raphe nucleus cell lines (Fig. 3). As in these other cell lines, this component also run as doublet in L6 myoblasts.

A distinct receptor complex was found on an embryonic mouse spinal cord motor neuron hybrid cell (Fig. 3). This line was obtained by fusion of E14 mouse spinal cord motor neurons and the N18TG2 mouse neuroblastoma, followed by selection of clones expressing high levels of choline acetyltransferase activity (Salazar-Gruesso et al., 2 *Neuroreport* 505, 1991). Importantly, SN6, a hybrid cell line of embryonic mouse basal forebrain cholinergic neurons and the same N18TG2 neuroblastoma (Hammond et al., 234 *Science* 1237, 1986), showed no GDNF receptors (Table I), indicating that the GDNF binding proteins seen on the motor neuron cell (hereafter referred to as MN-1) are likely to represent GDNF receptor components present in spinal motor neurons. As with the L6 myoblasts, the predominant receptor in MN-1 cells was preferentially cross-linked with EDAC, although in these cells it was a larger protein of 155kD (Fig. 3). This was subsequently identified to be a c-RET receptor (see Example 9 below). MN-1 cells also expressed 65-70kD binding proteins and low amounts of the 300kD receptor (Fig. 3 and Table I).

In order to dissect the individual constituent affinities of GDNF receptor

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subunits, displacement binding assays were performed, followed by cross-linking and SDS-PAGE. Receptor-ligand complexes were visualized by autoradiography. cut out from the gel and counted in a gamma counter. The resulting displacement curves indicated a K_d of approximately 0.2 nM for all components on RN33B and MN-1 cells (Fig. 4 a-c). These data at present do not clearly establish whether all GDNF receptor subunits display similar binding affinities or, whether they are all required to assemble a high affinity receptor complex.

Example 3

10 Biochemical characterization of GDNF receptors

The overall similarity in the pattern of receptors between GDNF and TGF- β prompted an examination of whether any of the previously identified receptors for TGF- β superfamily members was part of the GDNF receptor complex. Cross-linked ^{125}I -GDNF-receptor complexes from differentiated RN33B cells were subjected to immunoprecipitation with different anti-peptide antisera specific for all cloned TGF- β superfamily receptors, including type I receptors (ALK-1 to ALK-6), type II receptors TBR1, ActR1 and BMPR1, the type III receptors betaglycan, and endoglin. In a parallel control experiment, ^{125}I -TGF- β 1 was cross-linked to type I, type II and type III receptors on the mink lung epithelial cell line Mv1Lu followed by immunoprecipitation with antisera against TBR1 (ALK-5), TBR1 and betaglycan, respectively. Although type I, type II and type III TGF- β receptors were recovered in the control experiment, none of the GDNF receptor components in differentiated RN33B cells could be immunoprecipitated by any of the tested antisera (not shown). These data confirmed that the GDNF receptor subunits expressed on these cells are novel proteins.

Example 4

Endogenous GDNF expression in cell lines expressing GDNF receptors

Traditional models for the action of neurotrophic factors have described them as target-derived polypeptides that promote survival and differentiation of specific neuronal subpopulations. More recently, it has become evident that neurotrophic factors may also have paracrine and even autocrine modes of action

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(Ernfors and Persson, 3 *Eur. J. Neurosci.* 953, 1991; Acheson et al., 374 *Nature* 450, 1995). Expression of GDNF mRNA in cell lines expressing GDNF receptors was examined. Cells were homogenized in guanidine isothiocyanate (GITC) and β -mercaptoethanol. RNA extraction and GDNF RNase protection assay were as previously described (Trupp et al., *supra*).

Unexpectedly, all cell lines, with the exception of the motor neuron line MN-1, expressed substantial levels of GDNF mRNA as assayed by RNase protection analysis (Fig. 5). The highest GDNF mRNA expression was found in cells from raphe nucleus, which showed up to 5-fold higher expression than postnatal day 1 (P1) kidney, one of the richest sources of GDNF mRNA in the developing rat (Trupp et al., *supra*). Interestingly, upon differentiation of RN33B cells, GDNF mRNA expression decreased to about 30% of the level in undifferentiated cells (Figure 5). GDNF treatment of differentiated RN33B cells did not alter the expression of GDNF mRNA (Fig. 5) or GDNF receptors (not shown).

Expression of *c-ret* mRNA was investigated in RN33B, L6, and MN-1 cells, using the RNase protection assay. Ten micrograms of total RNA from the cell lines indicated was analyzed using a riboprobe complementary to 400 nucleotides of coding sequence from the kinase domain of the mouse *c-ret* mRNA. Although high expression was seen in MN-1 cells, no *c-ret* mRNA was detected in either the RN33B or L6 cells (Fig. 6). These results indicate that a signaling receptor for GDNF other than c-RET must be present in these cells.

Example 5

Activation of the ERK signal transduction pathway in GDNF responsive cell lines

Whether the GDNF binding proteins characterized in cell lines were able to form ligand-dependent signaling complexes was also investigated. Cell monolayers in 10 cm plates were incubated at 37°C in the presence of 50 ng/ml GDNF for the indicated time periods and immediately lysed with 1 ml of ice cold lysis buffer (as above) with the addition of 1 mM sodium orthovanadate. Whole cell lysates were fractionated by SDS-PAGE (10% polyacrylamide) and blotted to nitrocellulose

filters. Western blots were probed with an anti-phosphotyrosine antiserum (UBI, Lake Placed, NY), followed by horseradish peroxidase-conjugated goat anti-mouse IgG and developed with the ECL Western Detection System (Amersham, UK). For reprobing, blots were first stripped by a 30 minute incubation at 50°C in 62.5 mM Tris-HCl pH6.7, 100 mM β -mercaptoethanol, 2% sodium dodecyl sulphate. After removal of antibodies, blots were probed with a rabbit polyclonal antisera raised against recombinant rat ERK2 (a gift of Teri Boulton, Regeneron Pharmaceuticals Inc., Tarrytown, NY) which recognizes both ERK1 and ERK2, and developed as above using a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody.

Because of their distinct patterns of GDNF receptor subunits, intracellular signaling responses were initially characterized in the raphe nucleus cell line RN33B and in the motor neuron cell line MN-1. Changes in the pattern of tyrosine-phosphorylated proteins elicited by GDNF treatment of RN33B or MN-1 cells were investigated. Tyrosine phosphorylation is a universal mechanism of regulation of intracellular signaling proteins that is stimulated by numerous cytokines and growth factors. RN33B and MN-1 monolayers were exposed to a saturating concentration of GDNF (5 ng/ml) for different periods of time, and total cell lysates were analysed for tyrosine phosphorylation by SDS/PAGE and Western blotting with an anti-phosphotyrosine monoclonal antibody. Two proteins with mobilities corresponding to 42kD and 44kD, respectively, were phosphorylated on tyrosine within 5 minutes of GDNF treatment of RN33B cells (Fig. 7A). A similar result was obtained in differentiated RN33B cells (not shown) of exposure to GDNF.

Based on comparison of their size with descriptions of growth factor-induced protein tyrosine phosphorylation elsewhere (Qiu and Green, 9 Neuron 705, 1992), the 42kD and 44kD species would appear to be, respectively, p42 .k2 and p44 .k1 , two protein serine-threonine kinases members of the extracellular signal-regulated kinase (ERK, also termed microtubule-associated protein kinase) family (Boulton et al., 65 Cell 663, 1991). To confirm the identity of these proteins as ERK2 and ERK1, respectively, protein blots which had been reacted with the anti-phosphotyrosine antibody were stripped and reprobed with a rabbit polyclonal

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antibody raised against recombinant ERK2 that recognizes both ERK1 and ERK2 in protein blots. Comparison of autoradiograms of blots probed with the anti-phosphotyrosine antibody and the anti-ERK2 antibody identified the p42 and p44 proteins as ERK2 and ERK1, respectively (Fig. 7a). Although GDNF treatment of MN-1 cells appeared to only stimulate phosphorylation of ERK2, both ERK1 and ERK2 were present in MN-1 cell lysates (Fig. 7b). Thus, GDNF treatment stimulated very rapid and transient tyrosine phosphorylation of ERK1 and ERK2 in RN33B cells, but relatively slower and more sustained phosphorylation of ERK2 and MN-1 cells.

Activation of the ERK pathway has previously been shown to induce rapid and transient increase in transcription of immediate early genes, including the c-fos protooncogene (Gille et al., 358 *Nature* 414, 1992). Accordingly, the ability of GDNF to induce c-fos mRNA in differentiated raphe nucleus RN33B cells and in motor neuron MN-1 cells was investigated. For analysis of c-fos mRNA expression in cell lines, culture medium was changed 90 minutes prior to addition of 100 ng/ml GDNF to cell monolayers. At the indicated time intervals, media was removed, cells solubilized with guanidine isothiocyanate and β -mercaptoethanol and RNA extracted as previously described (Trupp et al., *supra*). Twenty micrograms of total RNA was fractionated on 1% agarose gels containing 0.7% formaldehyde and transferred to Hybond-C membranes (Amersham, UK). Northern blots were hybridized with an α - ^{32}P -dCTP labeled rat c-fos gene fragment (Curran et al., 2 *Oncogene* 79, 1987), washed at high stringency and visualized by autoradiography on x-ray films.

Cell monolayers were exposed to saturating concentrations of GDNF for different periods of time and levels of c-fos mRNA were subsequently analyzed in Northern blots of total RNA (Fig. 8). This analysis revealed transient upregulation of c-fos mRNA 15 minutes after exposure of RN33B cells to GDNF, returning to basal levels 45 minutes after treatment (Fig. 8a). c-fos mRNA was also upregulated in MN-1 cells but not until 30 minutes of GDNF treatment (Fig. 8b). Elevated c-fos mRNA levels persisted for about an hour and returned to basal levels 120 minutes after the initiation of treatment (Fig. 8b). Thus, like tyrosine phosphorylation of ERKS, c-fos mRNA upregulation induced by GDNF treatment

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was very rapid and transient in RN33B cells, but somewhat slower in MN-1 cells.

5 **Example 6**

Survival responses promoted by GDNF in differentiated raphe nucleus cells

Advantage was taken of the conditional nature of the immortalization of the raphe nucleus serotonergic cell line RN46A by examining whether GDNF may be a survival factor for differentiated raphe nucleus neurons. Survival assays were performed as previously described (Eaton et al., 1995, *supra*). Briefly, 10⁵ RN cells were seeded to collagen/fibronectin coated 8-well glass slides and incubated at 33°C (growth permissive temperature) until 75-90% confluent. The slides were then shifted to 39° (non-permissive temperature) and serum containing medium was replaced by B16 defined medium (Brewer and Cotman, 494 *Brain Res.* 65,1989) containing 1% BSA, 1 µg/ml transferrin, 5 µg/ml insulin, 100 mM putrescine, and nM progesterone plus or minus 0-50 ng/ml rhGDNF (Promega, Madison, WI). Media and GDNF were replaced every two days for 8 days after which the cells were fixed in 4% paraformaldehyde/2% glutaraldehyde, rinsed and coated with a glycerol mounting medium containing 1 mM bisbenzamide (Hoechst dye 33342) to stain viable nuclei. Fields of cells were magnified to 40x on a Zeiss Axiophot microscope, examined for fluorescent nuclei (at 355 nM excitation, 465 nM emission), the images video captured, and the cells counted with Imade I™ software. For each condition, 10 fields of cells were counted from each of 3 independent experiments.

RN46A cells were cultured at the non-permissive temperature in defined medium in the presence of increasing concentrations of GDNF. Nine days after plating, surviving cells were counted and compared with cultures established in the absence of GDNF. A 3-fold increase in the number of surviving cells was observed in cultures grown in the presence of GDNF (Fig. 9). The effect of GDNF on the survival of differentiated RN46A cells was dose dependent, with an EC50 at 5ng/ml.

Example 7**Generation, cloning and characterization of anti-GDNF monoclonal antibodies***Immunisation*

5 Five young female mice were immunised with 35 ug of insect cell-derived recombinant GDNF emulsified with complete Freund's adjuvant (FA). Second and third immunizations were performed 2 and 4 weeks after the first one in incomplete FA. All the injections were given intraperitoneally (i.p.). Two weeks after the last Immunisation, antibody titer in serum was checked by ELISA and Western Blot
10 analysis using standard methods. The mouse with the highest titer (more than 1:2000) was boosted i.p. with 3 μ g of GDNF in incomplete FA 3 days before the cell fusion.

Cell fusion

Cell fusion was done according to the method of Kohler and Milstein
15 (1975), incorporated herein by reference, with some modifications.

a) Day before fusion:

Viable cells from the Sp2/0 murine cell line were adjusted to 2×10^5 cells / ml with complete DMEM (10% fetal calf serum, 1% L-glutamine, 100 U/ml penicillin and 100 ug/ streptomycin sulphate).

20 Cells from a non-immunised mouse were obtained from the peritoneal cavity by injection of 0.34M sucrose solution. The cells were resuspended in complete DMEM containing: hypoxanthine 100 μ M; aminopterin 0.4M; and thymidine 16 μ M, (HAT medium), to 1×10^5 cells /ml. 100 μ l of the cell suspension was added to the 60 inner wells of 96 well plates and incubated overnight at 37°C in an
25 atmosphere of 5% CO₂ in air. These cells were the source of growth factors.

b) Fusion

Spleen cells from the mouse exhibiting the highest serum titer (see above) were homogenized in 10 ml DMEM removing surface fat and other adhering tissue in a sterile hood.

30 4.2×10^7 Sp2/0 cells were fused with 8.4×10^7 spleen cells in a solution of melted PEG (3000-3700, Hybri-Max, Sigma). The cells were then grown in HAT medium at 37° C in an atmosphere of 5% CO₂ in air. After one week of culture,

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the wells were inspected. When hybrids cells covered 10 to 50% of the surface area of the well, the culture supernatants were assayed for antibody by ELISA.

For the ELISA, wells of microplates (Costar, EIA/RIA plate high binding) were coated with 100 ul of 2ug/ ml of GDNF diluted in carbonate/ bicarbonate buffer, pH 9.6. After an overnight incubation at 4 °C, the wells were washed with 0.05M phosphate buffered saline, pH 7.2, containing 0.05% Tween (PBS-T). Nonspecific binding was blocked with PBS-T containing 3% non-fatty milk and 1% goat normal serum. Supernatant samples were incubated 4 hours at room temperature. Peroxidase goat anti-mouse antibody was used and the substrate was o-phenylenediamine dihydrochloride (OPD). Plates were read at 492 nm in an ELISA reader. Negative controls included completed medium and normal mouse serum.

The hybrids were grown in HAT medium up to two weeks after fusion. Cells were subsequently grown in HT medium until the completion of two cloning procedures, using the limiting dilution method. After each step (when cells reached 10 to 50% confluence), assays for specific antibody in supernatants were done by ELISA. Upon recloning, 5 positive hybridoma clones were chosen and the cells were maintained in complete DMEM for 30 days.

Isotyping of monoclonal antibodies

The class and subclass of the monoclonal antibodies were determined by ELISA using a DAKO panel for isotyping of mouse monoclonal antibodies. All five 5 monoclonal antibodies were characterized as IgG₁.

Purification of monoclonal antibodies

Monoclonal antibodies from culture supernatants were purified by Protein G Sepharose fast flow (Pharmacia, Biotech) according to manufacturer's instructions. Culture supernatants were concentrated and filtered through a 0.45 µm membrane (Schleicher and, Schull, Germany) and then pumped overnight through the column previously equilibrated with 20mM sodium phosphate, pH 7.0. Ig was eluted with 0.05M glycine buffer.

Example 8

A motor neuron cell line showing biological and biochemical responses to

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GDNF

MN-1 cell monolayers were exposed to increasing concentrations of GDNF in serum-free medium and assayed 3 days later for cell survival and growth by measurement of acid phosphatase activity (Clontech). GDNF was produced and purified from baculovirus infected insect cells as previously described (Trupp et al., *supra*). GDNF treatment of serum deprived-MN-1 monolayers increased cell number in a dose-dependent manner (Fig. 10a). The biological response of MN-1 cells correlated with biochemical and transcriptional responses to GDNF treatment. MN-1 cell monolayers were exposed to 50 ng/ml GDNF for increasing periods of time, cell lysates were fractionated by SDS/PAGE and Western blots probed with an anti-phosphotyrosine antibody (UBI).

Several proteins were seen to have increased tyrosine phosphorylation after GDNF treatment of MN1 cells, including a protein with an electrophoretic mobility of 42K (Fig. 10b). Based on comparison of its size with descriptions of growth factor-induced protein tyrosine phosphorylation elsewhere (Boulton, T.G., *et al. Cell* 65, 663-75 (1991), the 42K species would appear to be p42^{erk2}, a serine-threonine kinase member of the extracellular signal-regulated kinase (ERK) family. The identity of this protein as ERK2 was confirmed after immunoprecipitation with an antiERK2 polyclonal antiserum followed by analysis of tyrosine phosphorylation (Fig. 10c). Lysates of GDNF-stimulated MN-1 cells were immunoprecipitated with an anti-ERK2 antiserum (Santa Cruz) that also recognizes ERK1 followed by antiphosphotyrosine Western blotting. This analysis further revealed that another member of the ERK family, p44erk1, was also phosphorylated on tyrosine after GDNF treatment of MN-1 cells (Fig. 10c). Activation of the ERK pathway has previously been shown to induce a rapid and transient increase in the transcription of immediate early genes, including the *c-fos* proto-oncogene (Gille et al., *Nature* 358, 414-7 (1992).

Example 9

The product of the *c-ret* proto-oncogene as a signal transducing receptor for GDNF

GDNF receptor complexes from MN-1 cells could be recovered by

immunoprecipitation with anti-GDNF antibodies or by binding to lectin-Sepharose beads (Fig. 11a). Unexpectedly, the 180kD receptor complex (i.e., c-RET; 180kD - 23kD = 157kD, which is approximately equal to the 155kD receptor identified as c-RET -- see Example 2, *infra*) could also be recovered by immunoprecipitation
5 with anti-phosphotyrosine antibodies (Fig. 11a), indicating that the GDNF binding protein in this complex could be a receptor tyrosine kinase.

The product of the *c-ret* proto-oncogene is highly expressed in primary motor neurons (Pachnis et al., *supra*, and Tsuzuki, T., *et al. Oncogene 10*, 191-8 (1995) and is of similar molecular weight as the major GDNF receptor component
10 detected in MN-1 cells (Takahashi, M., *et al. Oncogene 3*, 571-578 (1988). We tested whether this species represented a C-RET-GDNF cross-linked complex by immunoprecipitation with anti-c-RET antibodies.

¹²⁵I-GDNF was cross-linked to MN-1 cells using EDAC and receptor complexes were precipitated with antibodies against GDNF (Trupp et al., *supra*),
15 lectin Sepharose beads (Formica), anti-phosphotyrosine antibodies (UBI), anti-c-RET antibodies (Santa Cruz) and control antibodies from non-immune rabbits. An antipeptide c-RET rabbit antiserum readily immunoprecipitated the major 180kD ligand-receptor complex in MN-1 cells (Fig. 11a), while a number of unrelated monoclonal and polyclonal antibodies used as controls failed to immunoprecipitate
20 this complex (Fig. 11 a and data not shown).

Because the product of the *c-ret* gene is a receptor tyrosine kinase, we investigated whether GDNF could stimulate tyrosine phosphorylation of the c-RET protein in MN-1 cells. MN-1 cell monolayers were exposed to GDNF at different concentrations or for different periods of time and cell lysates were
25 immunoprecipitated with anti-c-RET antibodies and analyzed by SDS/PAGE and Western blotting with antiphosphotyrosine antibodies as disclosed above. GDNF treatment stimulated rapid c-RET tyrosine phosphorylation in MN-1 cells (Fig. 11b). Maximal phosphorylation was reached 5 minutes after GDNF treatment and lasted for at least 60 minutes. A dose-response analysis of GDNF induced c-RET
30 phosphorylation in MN-1 cells showed maximal phosphorylation at 30 ng/ml of GDNF (Fig. 11b), which is similar to the response of both serum deprived MN-1 cells (Fig. 10a) and embryonic sympathetic neurons (Trupp et al., *supra*) to

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GDNF. Taken together, these data indicate that the c-RET receptor may be an important component in the signal transduction mechanism of GDNF.

Example 10

5 *c-ret* transfection reconstitutes GDNF binding and biological activities to GDNF

Experiments were conducted to determine whether expression of the *c-ret* gene product could be sufficient to allow binding of GDNF to cells lacking GDNF receptors. To this end, GDNF binding and cross-linking experiments were performed in naive 3T3 fibroblasts, and 3T3 cells stably transfected with either a
10 wild type *c-ret* or an oncogenic form of this gene found in MEN2a patients (Mulligan et al., *supra*). For *c-ret* expression in transfected cells, human wild type *c-ret* and MEN2a-ret cDNAs were subcloned in pcDNA3 (Invitrogen). Cold GDNF was used at 50x molar excess. For survival/growth assays, cells were cultured for 6 days in serum-free medium supplemented with the indicated concentrations of
15 GDNF; medium and GDNF were replaced every two days. Cell number was quantified by measurement of acid phosphatase activity (Clontech).

After immunoprecipitation with c-RET antibodies, GDNF-labeled receptor complexes of approximately 180K were detected in both MEN2a-ret and *c-ret* transfected 3T3 fibroblasts, but not in untransfected cells (Fig. 12a). The labeling
20 could be displaced by excess cold GDNF, indicating that it represented specific GDNF binding (Fig. 12a).

Experiments were also conducted to determine whether *c-ret* could mediate a biological response to GDNF upon transfection in non-responsive cells. Survival and growth responses to GDNF were investigated in untransfected and *c-ret*
25 transfected 3T3 fibroblasts cultured in serum-free medium. GDNF elicited a dose-dependent increase in cell number in *c-ret* transfected, but not in untransfected, 3T3 cells (Fig. 12b) which was comparable to the one previously observed in serum-deprived MN-1 cells. Since naive 3T3 cells did not express any appreciable amount of GDNF receptors prior to transfection (see Fig. 12a), it was concluded
30 that *c-ret* expression was sufficient for mediating a biological response to GDNF in these cells.

Example 11***c-ret* expression in adult brain and dopaminergic neurons of the substantia nigra**

Experiments were conducted to determine whether the *c-ret* product may mediate the neurotrophic effects of GDNF in the brain by examining the expression of *c-ret* in different regions of the rat central nervous system. A rat *c-ret* riboprobe was generated using as template a cDNA fragment obtained by PCR with primers based on sequences U22513 and U22514 (Genbank accession numbers). High levels of *c-ret* mRNA were found in MN-1 cells and in rat spinal cord (data not shown). High *c-ret* mRNA expression was also found in the adult pons, medulla, locus coeruleus and hypothalamus (Fig. 13a), as well as in thalamus and cerebellum (data not shown). *c-ret* mRNA was expressed at barely detectable levels in striatum, hippocampus and cerebral cortex (Fig. 13a). In the ventral mesencephalon, containing the cell bodies of GDNF-responsive dopaminergic neurons, *c-ret* mRNA levels increased progressively during post-natal development (Fig. 13b). A peak of expression was detected between post natal day 6 (P6) and P8, at which time axons of dopaminergic neurons of the substantia nigra begin innervation of the striatum, and coincident with an increase in GDNF mRNA expression in this target region (Fig. 13b). For mRNA quantification, a glyceraldehyde-3-P dehydrogenase (GAPDH) riboprobe was included in the RPA, and values of relative mRNA expression, obtained after densitometric scanning of gel autoradiograms, were normalised using the GAPDH signal of each RNA sample. RPA for GDNF mRNA has been previously described (Trupp et al., *supra*).

In situ hybridisation and immunohistochemistry were performed as previously described (Arenas, E. & Persson, H. *Nature* 367, 368-371 (1994); Neveu, I & Arenas, E. *J. Cell Biol.* in press (1996). c-RET protein was detected using a hamster monoclonal anti-mouse c-RET antibody which also recognises rat c-RET (Lo, *supra*) followed by fluorescein-conjugated rabbit anti-hamster secondary antibodies (Southern Biotechnologies). *In situ* hybridization on sections through the adult substantia nigra revealed strong labelling over neurons throughout this structure (Figs. 14 a-b). In addition, cells positive for c-RET-like immunoreactivity (c-RET-LI) were found throughout the adult substantia nigra.

with strong labelling over cell bodies (Fig. 14c).

In order to establish that *c-ret* expression in the adult substantia nigra was confined to dopaminergic neurons, these cells were selectively lesioned with a unilateral injection of 6-hydroxydopamine (6-OHDA); the cells were then analyzed for *c-ret* mRNA expression by *in situ* hybridisation. Lesions of dopaminergic neurons of the substantia nigra were performed by stereotaxic injections of 8 µg 6-OHDA in the medial forebrain bundle at the following coordinates: 1.6 mm caudal to bregma, 1.3 mm lateral to midline, and 8.4 mm under the dural surface with the incisor bar 5 mm over the interaural line. Animals were pretreated with 25 mg/kg desipramine (i.p.) 30 minutes prior to 6-OHDA injection. 0.75×10^6 GDNF-expressing fibroblast cells in 3 µl of medium were injected supranigrally at the following coordinates: 3.1 mm from interaural line, 2 mm lateral to midline, and 7 mm under the dural surface, with the incisor bar at -3.3 mm. Lesion and grafting in the locus coeruleus were as previously described (Arenas et al., *Neuron* 15, 1465-1473 (1995)). The generation and characterisation of GDNF expressing fibroblasts have been described previously (Arenas et al., supra).

Five hours after the lesion, no difference could be seen between ipsi and contralateral sides in *c-ret* mRNA expression (Fig. 14d). However, a marked reduction in *c-ret* mRNA expression was seen in the lesioned substantia nigra already one day after 6-OHDA treatment, and was nearly absent 5 days after the lesion (Fig. 14d). *c-ret* mRNA expression in the side contralateral to the lesion was, however, not affected (Fig. 14d). This result indicated that in the adult substantia nigra, *c-ret* mRNA expression was confined to dopaminergic neurons.

Example 12

GDNF rescues c-RET-positive dopaminergic and noradrenergic neurons

Experiments were conducted to determine whether c-RET expressing neurons of the adult substantia nigra and locus coeruleus responded to GDNF. For this, nigral dopaminergic neurons lesioned with 6-OHDA, and were then examined to determine whether grafts of GDNF expressing fibroblasts induced responses on c-RET immunoreactive neurons. In lesioned animals that received a graft of control fibroblasts, no c-RET-LI could be detected, indicating a depletion of *c-ret*-

expressing cells by selective lesion of dopaminergic neurons in the adult substantia nigra (Fig. 14e). However, c-RET-LI could be rescued by the GDNF-expressing graft, where c-RET immunopositive fibers could be seen surrounding and penetrating the graft (Fig. 14f). Similar results were obtained in the locus coeruleus, where lesion with 6-OHDA depleted c-RET-immunoreactive cell bodies (Fig. 14g), which could be rescued by exogenous administration of GDNF (Fig. 14h). In both brain regions, the rescue of c-RET-LI positive cells and sprouting in the animals grafted with GDNF-expressing fibroblasts paralleled that of tyrosine hydroxylase immunoreactivity (data not shown), demonstrating that c-RET-expressing adult dopaminergic and noradrenergic neurons respond to GDNF.

Example 13

Identification of GDNF c-RET receptors

PC12 cells and NB2/a cells were washed three times with serum free RPMI-1640 or DMEM, respectively, plated on noncoated (NB2/a cells) or collagen-coated (PC12 cells) dishes (5000-6000 cells per dish) in the presence or absence of 50 ng/ml of GDNF (Peprotech EC Ltd.) and the number of cells was microscopically counted after 48 hours. PC12 and NB2/a cells were harvested (100,000 cells, five parallels), incubated with 10 ng/ml human ¹²⁵I-GDNF (iodinated by Chloramine T method, 100 μ Ci/ μ g) in the presence or absence of 50-fold unlabeled GDNF for 120-150 min on ice, the unbound factor was removed by centrifugation through 30% sucrose cushion, and the cell-associated radioactivity counted on 1271 RIAGAMMA counter (LKB Wallac).

Recombinant human GDNF promoted survival of about 20% of serum deprived rat pheochromocytoma PC12 cells at concentration of 50 ng/ml (Fig. 15a). Serum-deprived PC12 cells are also maintained by nerve growth factor (NGF). Upon treatment with (NGF), PC12 cells also stop dividing and differentiate into sympathetic neuron-like cells with long neurites. Thus, GDNF is a survival-promoting factor for PC12 cells, although less potent than NGF, but it does not induce differentiation of PC12 cells at the concentrations studied, presumably because of the differences in signal transduction of NGF-activated trkA receptors and GDNF receptors.

Human neuroblastoma NB2/a cells were plated in serum-free medium in the presence or absence of 50 ng/ml of GDNF and the number of cells was counted after 48 hr of culture. GDNF significantly increased the number of NB2/a cells (Fig. 15b). Monkey COS cells, human SY5Y cells and mouse NIH 3T3 cells showed neither mitogenic nor survival response to GDNF (data not shown). Thus, GDNF exerts biological effects on rat PC12 cells and human NB2/a cells, indicating that both cell lines express functional GDNF receptors.

To determine, whether GDNF binds to the responsive cells, PC12 cells and NB2/a cells were incubated with ^{125}I -labeled human GDNF at 40°C as indicated in the legend to Fig. 15. As shown in Fig. 15c, both PC 12 and NB2/a cell lines bind GDNF 30 efficiently. More importantly, the binding of ^{125}I -labeled GDNF could be competed with a 50-fold excess of unlabeled GDNF (Fig. 15b). Thus, the binding of GDNF to the receptors on PC12 and NB2/a cells appears to be specific.

Example 14

Identification of GDNF c-RET binding components

PC12 cells, SY5Y neuroblastoma cells and NB2/a cells were chemically cross-linked to ^{125}I -GDNF with EDC. 3-5 x 10⁶ cells or mechanically dissociated cells from 2 E20 rat kidneys were incubated with 10 ng/ml of ^{125}I -GDNF for 1 hour on ice and cross linked with 30 mM EDAC (Pierce) for 30 minutes on ice. Detergent lysates were immunoprecipitated, the precipitates collected by Protein A-Sepharose, separated on 7% SDS-PAGE, and visualized by Phosphorimager SI (Molecular Dynamics).

The resulting complexes were immunoprecipitated with rabbit antibodies to GDNF, analyzed by SDS-PAGE and visualized by autoradiography. Embryonic kidney cells were also studied as the source of putative GDNF receptor (Suvanto, P. et. al., *Eur. J. Neurosci.*, 8, 101-107 (1996); Sainio, K. et. al., *Nature*, (1996) submitted). Cross-linked complexes of 170 and 190 kD were obtained from the extracts of PC12 cells, SY5Y cells and NB2/a cells and a 190 kD complex from embryonic kidney extracts. (Fig. 16).

The molecular weights of the crosslinked proteins minus GDNF of approximately 25-30 kD substantially, if not exactly, correspond to the molecular

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weights of c-RET protooncogene, an orphan receptor tyrosine kinase (Takahashi, M., Ritz, J. & Cooper, G. M. *Cell*, 42, 581-588, 1985; Takahashi, M. et.al., *Oncogene*, 3, 571-578 (1988)) (140 kD and 160 kD, representing differently glycosylated forms of c-RET., Tsuzuki, T., Takahashi, M., Asai, N., Iwashita, T., Matsuyama, M. & Asai, J. *Oncogene*, 10, 191-198 (1995).

Example 15

Affinity Cross Linking of GDNF to c-RET

The cross linked complexes were immunoprecipitated from the NB2/a cells with the cocktail of antibodies recognizing extracellular and intracellular part of the c-RET receptor. As shown in Fig. 17a (lane 1), the complexes of 170 kD and 190 kD were precipitated by anti-c-RET antibodies, which thus correspond to cross linked GDNFc-RET complexes. Binding of ¹²⁵I-GDNF to c-RET proteins was completely abolished by 500-fold excess of unlabeled GDNF (lane 2). No proteins were precipitated by monoclonal anti-neurofilament antibodies (lane 3) or by Protein A-Sepharose only (lane 4). No cross linked complexes were obtained from COS cells (not shown). Since *c-ret* proto-oncogene is a glycoprotein, ¹²⁵I-labeled NB2/a cell extracts were also immunoprecipitated with wheat germ agglutinin. Again, proteins of 170 and 190 kD were obtained (lane 5).

To establish further that GDNF specifically binds c-RET, the mouse *c-ret* cDNA was cloned into the mammalian expression vector PBK-CMV and transiently expressed in monkey COS cells. Mouse *c-ret* cDNA (Pachnis, V., Mankoo, B. & Costantini, F. *Development*, 119, 1005-1017 (1993)) in pbluescript SK' (Stratagene) was cleaved with *SacII* and *EcoRV* and cloned into *SacII* and *SmaI* site of pBK-CNV vector (Stratagene). COS cells were transiently transfected with *c-ret* cDNA or with empty plasmid by electroporation (Bio Rad) with ~ 30 % efficiency by fluorescence of cotransfected Red Shift Green Fluorescent Protein in PEF-BOS vector. 48 hours later, 10 x 10⁶ transfected COS cells or 3-5 x 10⁶ parental COS cells or NB2/a cells were treated With ¹²⁵I-GDNF, cross linked and analysed as specified in legends of Fig. 15 and Fig. 16.

First, the expression of c-RET protein by was examined Western blotting. *c-ret*-transfected COS cells (Fig. 18a) and NB2/a cells (not shown) expressed

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detectable amounts of the c-RET protein, whereas no c-RET protein was detected in mock-transfected (with PBK-CMV plasmid) COS cells (Fig. 18a). PC12 cells also express c-RET protein, albeit at considerably lower level than NB2/a cells or *c-ret*-transfected COS cells (not shown). COS cells, transiently expressing mouse *c-ret* proto-oncogene were incubated with ^{125}I -GDNF. As shown in Fig. 17b, those cells bound GDNF, and binding of ^{125}I -GDNF can be competed with excess of unlabeled GDNF. In contrast, no significant binding of GDNF was observed in mock-transfected COS cells.

10 Example 16

Phosphorylation of tyrosine residues

10 X 10^6 transfected COS cells (48 hr after transfection) were treated with 50 ng/ml of GDNF (Preprotech EC Ltd.) for 5 minutes in serum-free DMEM, or not treated, and then quickly washed with the same medium. NB2/a cells were similarly treated (results not shown). c-RET proteins were immunoprecipitated from detergent extracts by cocktail of monoclonal (Lo, L. & Anderson, D. J. *Neuron*, 15, 527-539 (1995) and polyclonal (Santa Cruz) anti-c-ret antibodies, separated by 7% SDS-PAGE, transferred to nitrocellulose, probed by anti-c-ret antibodies (Santa Cruz), stripped and reprobed by anti-phosphotyrosine antibodies (Sigma).

This treatment resulted in significant increase in tyrosine phosphorylation of 190 kD cRET proto-oncogene, the 170 kD form being less prominently phosphorylated (Fig. 18b). In both cell lines, relatively high c-RET phosphorylation was detected also in the absence of GDNF (Fig. 18), most probably via endogenous GDNF secreted by these cells and/or ligand-independent receptor dimerization.

Example 17

30 ^{125}I -GDNF binds to c-ret-positive enteric neurons

^{125}I -GDNF was bound to developing rat tissue explants *in situ*. *In situ* binding of human ^{125}I -GDNF (PeproTech. EC Ltd.), iodinated by Chloramine T

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Method, was carried out essentially as described (Partanen and Thesleff, 1987). Briefly, explants of E15 rat gut were incubated with 10 ng/ml of ^{125}I -GDNF in Eagle's minimal essential medium on the Nuclepore filter (Costar) for 90 min at room temperature. 250-fold excess of unlabeled GDNF was applied as a competitor to control explants. After careful washing, the explants were fixed with 3.5% paraformaldehyde in PBS, sectioned and exposed to NTB-2 emulsion (Kodak).

The gastrointestinal tract was chosen as it strongly expresses GDNF mRNA (Suvanto *et al.*, 1996); Figure 19 a and b) and c-RET-positive neurons are absent in the gastrointestinal tract in *c-ret*-deficient mice (Schuchart *et al.*, 1994; Durbec *et al.*, 1996). ^{125}I -GDNF binds to a group of cells within the muscle layer of embryonic day (E)15 rat gut (Figure 19 c and d). This binding was specific as it was totally competed with 250-fold excess of unlabeled GDNF (Figure 19h). The cells that bind GDNF were the enteric neurons of the myenteric plexus, as revealed by peripherin immunoreactivity (Figure 19f). Moreover, these neurons also expressed *c-ret* mRNA, as demonstrated by *in situ* hybridization (Figure 19e).

Cloning of the GDNF cDNA and *in situ* hybridization with GDNF probe was performed exactly as described (Suvanto *et al.*, 1996). A 646 bp long fragment of mouse *c-ret* cDNA (Pachnis *et al.*, 1993) covering the 3'-region of the shorter form (Takahashi *et al.*, 1988) of *c-ret* was cloned into *NotI-XhoI* site of pBSK+ vector (Stratagene). cRNAs in antisense and sense orientation were labeled with digoxigenin-UTP (Boehringer-Mannheim), hybridized to cryosections through E15 rat gut and visualized with alkaline phosphatase-conjugated anti-digoxigenin antibodies according to manufacturers instructions. In both cases, only background labeling was obtained with hybridization of corresponding probes in sense orientation (Figure 19g). Polyclonal anti-peripherin antibodies (Bio-Rad) were applied to cryosections of E15 rat gut at a dilution of 1:100 for 1 hr and visualized by FITC-conjugated secondary antibodies (Jackson). Thus, GDNF specifically binds to c-RET-expressing enteric neurons of developing rat.

Example 18

Affinity-crosslinking of GDNF to c-RET

PC12 cells and NB2/a cells were washed three times with serum-free RPMI-1640 or DMEM, respectively, plated on uncoated (NB2/a cells) or collagen-coated (PC12 cells) dishes (5000-6000 cells per dish in triplicate) in the presence or absence of 50 ng/ml of GDNF (PeproTech EC Ltd.), and the number of cells microscopically counted after 48 h.

For c-RET expression in transfected cells, the shorter form (Takahashi et al., 1988) of human wild-type *c-ret* cDNA was subcloned in pcDNA3 (Invitrogen). 3T3 fibroblasts were stably transfected with c-ret expression plasmid or with empty vector (mock-transfected cells) and positive cells lines selected with G418.

Transient transfection of trkC 3T3 fibroblasts (Ip et al. (1993) *Neuron*, 10,137-149) with human *c-ret* cDNA in pcDNA3 vector or with empty vector was performed by the lipofectin method (Gibco-BRL). c-ret and mock-transfected cells (10.000 - 15.000 cells per well) in five parallels were treated with rat GDNF (Trupp et al. (1995) *J. Cell. Biol.* 130, 137-148) at indicated concentrations for five days. NT-3 was used as positive control at 30 ng/ml. Cell number was quantified by measurement of acid phosphatase activity using Abacus™ Cell Proliferation Kit (Clontech).

3-5 x 10⁶ PC12 cells, NB2/a cells, COS cells or *c-ret*-3T3 as well as mock-3T3 cells or mechanically dissociated cells from two F20 or from 17 E15 rat kidneys were incubated with 10 ng/ml of ¹²⁵I-GDNF (human GDNF from PeproTech EC Ltd. or rat GDNF from C. F. Ibanez) (Trupp et al., 1995), iodinated by Chloramine T method, for 1 hour on ice. 250-fold excess of unlabeled GDNF (PeproTech EC) or TGF-β1 (kindly provided by Dr. M. Laiho) was applied to control sample. ¹²⁵I-GDNF was then crosslinked to the cells with 30 mM of ethyl-dimethylaminopropyl carbodiimide (EDAC) (Pierce) for 30 minutes on ice. Detergent lysates of the cells were immunoprecipitated with polyclonal anti-GDNF antibodies (Santa Cruz) or with the cocktail of monoclonal (kindly provided by Dr. D. Anderson, Lo and Anderson, 1995) and polyclonal (Santa Cruz) anti-c-RET antibodies to neurofilament proteins (a gift of Dr. I. Virtanen) were used as control antibodies. The precipitates were collected by Protein A-Sepharose (Pharmacia) or by WGA-agarose (a gift from Dr. O Renkonen), separated on 7% SDS-PAGE, and visualized with a Phosphorimager SI (Molecular DynanLics).

First, ^{125}I -GDNF was crosslinked to PC12 cells, NB2/a cells and COS cells with ethyl-dimethylaminopropyl carbodiimide (EDAC), and the complexes were precipitated with anti-GDNF antibodies. As shown on Figure 20a, complexes with molecular weight of 190 kD and 170 kD were obtained from PC12 and NB2/A
5 cells, but not from COS cells. The molecular weights of the cross linked proteins (minus GDNF monomer of $\sim 25\text{K}$) correspond to those of c-RET, (140 kD and 160 kD, representing partially and fully glycosylated isoforms of c-RET, respectively) (Takahashi *et al.*, 1988).

Next, ^{125}I -GDNF was crosslinked to PC12 and NB2/a cells by EDAC and immunoprecipitated formed complexes with anti-c-RET antibodies. The bands with
10 molecular weight of 190 kD were obtained from both cell lines (Figure 20 a and b). Formation of the complexes was abolished by 500-fold excess of unlabeled GDNF. The reason why both fully and partially glycosylated forms of c-RET were precipitated by anti-GDNF antibodies, but only the larger isoform by anti-c-RET
15 antibodies, is unclear. The same complexes, although much weaker, were also obtained when dithiobis(succinimidylpropionate) was used as a crosslinker (data not shown).

The EDAC-crosslink approach was also used to reveal GDNF-c-RET complexes from E15 embryonic kidney cells, where c-ret mRNA is strongly
20 expressed in the tips of growing ureter branches. With both anti-GDNF and anti-c-RET antibodies, a band of 190 kD was obtained (Figure 20 a and b) that was competed with excess of unlabeled GDNF. Thus, only the fully glycosylated form of c-RET is expressed in embryonic kidney cells.

Crosslinked ^{125}I -GDNF-c-RET complexes from the cells ectopically
25 expressing *c-ret* were also demonstrated. 3T3 cells were transfected with *c-ret* cDNA or with empty plasmid, and established stable transfected cell lines (*c-ret*-3T3 cells or mock-3T3 cells). Cross linking of ^{125}I -GDNF to these cells followed by anti-RET-precipitation revealed a 190 kD band that was abolished with 250-fold excess of unlabeled GDNF (Figure 20b). As GDNF is a distant member of TGF- β
30 family, we also used a 250-fold excess of TGF- β 1 as a competitor. No competition was observed with TGF- β 1 (Figure 20b) Taken together, these data show that GDNF directly and specifically binds to c-RET.

Example 19**GDNF specifically increases tyrosine phosphorylation of c-RET**

c-ret-3T3 cells and mock-3T3 cells were treated with GDNF and the proteins from these cells were immunoprecipitated with anti-c-RET antibodies. The precipitated proteins were then analyzed by Western blotting with anti-phosphotyrosine antibodies. 10 x 10⁶ *c-ret*-3T3 cells were treated with different doses of GDNF (PeproTech LC Ltd. or from C. F. Ibanez) (Trupp *et al.*, 1995) for 5 min, or with 50 ng/ml of GDNF for indicated times in serum-free Dulbecco's modified Eagle's medium containing 1 mM Na₃VO₄ and then quickly washed with the same medium. c-RET proteins were immunoprecipitated from detergent extracts, containing 1 mM Na₃VO₄ by cocktail of monoclonal (Lo, L. and Anderson, D.J. (1995) *Neuron*, 15 527-539) and polyclonal (Santa Cruz) anti-c-RET antibodies, separated by 7% SDS-PAGE and transferred to nitrocellulose which were probed by anti-phosphotyrosine antibodies to nitrocellulose with was probed by anti-phosphotyrosine antibodies PY20 (Transduction Laboratories), then stripped and reprobed by anti-c-RET antibodies (Santa Cruz).

As shown on Figure 21a, a short treatment of *c-ret*-3T3 cells with GDNF dose-dependently (beginning at 25 ng/ml) increased tyrosine phosphorylation of the 160 kD c-RET isoform, whereas the phosphorylation of the 140 kD isoform remained unchanged. An increase in c-RET phosphorylation was evident at 25 ng/ml of GDNF and above it. No c-RET proteins were detected in mock-3T3 cells. *c-ret*-3T3 cells were also treated with GDNF (50 ng/ml) for different times. An increase in c-RET tyrosine phosphorylation was evident after 5 minutes of treatment and continued at least for one hour (Figure 21b). With prolonged exposition, the increase in phosphorylation of lower c-RET isoform also became evident. A basal level of c-RET phosphorylation was detected in the absence of GDNF, possibly via a ligand-independent dimerization of that receptor. To reveal the amounts of c-RET protein in these experiments, the filters were stripped from antibodies and reprobed with anti-c-RET antibodies. The level of c-RET protein was not changed by GDNF treatment in *c-ret*-3T3 cells (Figure 21a and b, lower panels). The finding that GDNF specifically activates c-RET indicates that c-RET is a signaling receptor for GDNF.

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Example 20**c-RET expression confers GDNF-responsiveness to 3T3 cells**

Mouse 3T3 fibroblast cell line expressing *trkC* (*trkC*-3T3) (Ip *et al.* 1993) were transiently transformed with *c-ret* expression plasmid. *trkC*-3T3 cells die
5 within 2-3 days in serum-free medium in the absence of *trkC* ligand neurotrophin-3 (NT-3) (Ip *et al.*, 1993) and do not express detectable amounts of *c-ret*. GDNF dose-dependently increased the number of *c-ret*-transfected but not of mock-transfected *trkC*-3T3 cells (Figure 22), which was comparable to the response elicited by NT-3. Whether this is a proliferative or survival-promoting response
10 could not be distinguished based upon the data. Thus, introduction of c-RET to GDNF-nonresponsive cells is sufficient to bring about the biological response to GDNF.

15

Example 21**Isolation of GDNF receptor**

L6 myoblast cells were lysed with 1% NP40 and cell lysates were fractionated by anionic exchange on a Q-Sepharose column. Fractions eluted at
20 different ionic strength were dialyzed and assayed for binding to GDNF immobilized on a chip in a Biacore device (Pharmacia). A distinct binding component was detected in a fraction of L6 cell lysates (Figure 23a). In the Figure, solid bars indicate total protein (as absorbance at 280 nm); hatched bars indicate GDNF binding (in resonance units). This fraction was not particularly rich
25 in protein, indicating a substantial purification over the total protein mixture. The equivalent fraction of a COS cell lysate did not show binding under the same conditions (data not shown).

Further purification of the GDNF binding activity in the first 1M salt fraction was obtained after hydrophobic interaction chromatography (Figure 23b).
30 The data represents the ratio between GDNF binding (in resonance units) and protein concentration (OD at 280 nm). Fractions were eluted with a step-wise gradient of ammonium sulfate.

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Alternatively, purification may be effected by crosslinking GDNF to cells in the presence of tracer amounts of radiolabeled ligand, and ligand/receptor complexes can be fractionated through ion exchange chromatography followed by hydrophobic interaction chromatography and SDS/PAGE. Bands corresponding to the molecular weights of GDNF-receptor complexes can be excised, dissociated, and then sequenced by mass spectrometry or Edman degradation, depending upon the yield of recovery.

Example 22

10 A novel GDNF-binding protein in brain

By ligand blotting, we have identified another GDNF-binding protein from total brain extract. We bound ^{125}I -GDNF to the filters carrying protein from the total extracts of brain and liver (a ligand blot assay). A major band with MW of about 50 kD was obtained from brain extract, but not from liver (Figure 24). This binding is specific as ^{125}I -GDNF did not bind to other proteins from total lysates, it is not found in liver lysates (nor in some other tissues), and it can be competed with excess of unlabeled GDNF. Binding of ^{125}I -GDNF to c-RET was not revealed in the ligand blots. The reason for this may be the very low share of c-RET in total brain extract. Alternatively, by analogy with other receptors for GDNF, c-RET might not bind GDNF directly, but might first bind to another nonsignalling receptor that thereafter presents the ligand to c-RET, a signaling receptor. A 50 kD GDNF-binding protein is a good candidate for the putative presenting receptor.

25 Example 23

Protocol for isolating novel signaling receptors for GDNF

In the absence of serum, 3T3 fibroblasts can be made dependent on a given exogenous growth factor provided appropriate receptors are expressed on the cell surface. An expression library can be made using RN33B cDNA, which can then be transfected into 3T3 fibroblasts by procedures well known in the art (Maniatis et al., *supra*). Stable transfectants can be selected in serum-free media supplemented with GDNF. Fibroblast clones that express signaling GDNF receptors will

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selectively grow in the presence of GDNF in serum-free media. The selection step may allow detection of even very rare clones due to their differential growth advantage. Further analysis of the recovered clones in media with or without GDNF would help to distinguish GDNF-dependent from GDNF-independent survival of clones.

5

All references cited herein are hereby incorporated by reference in their entireties.

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WHAT IS CLAIMED IS:

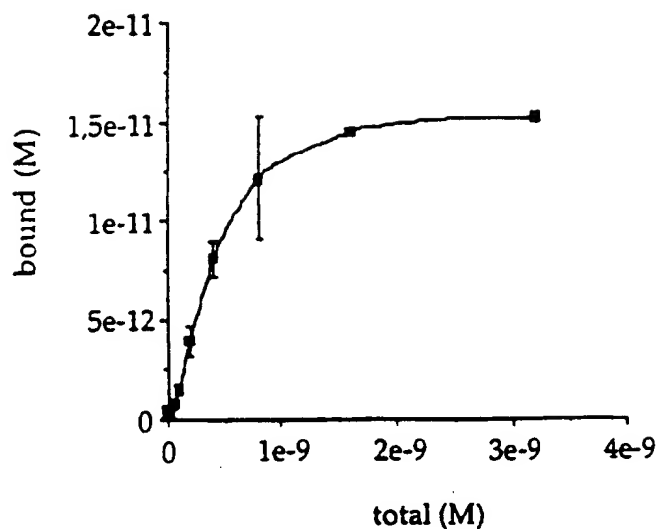
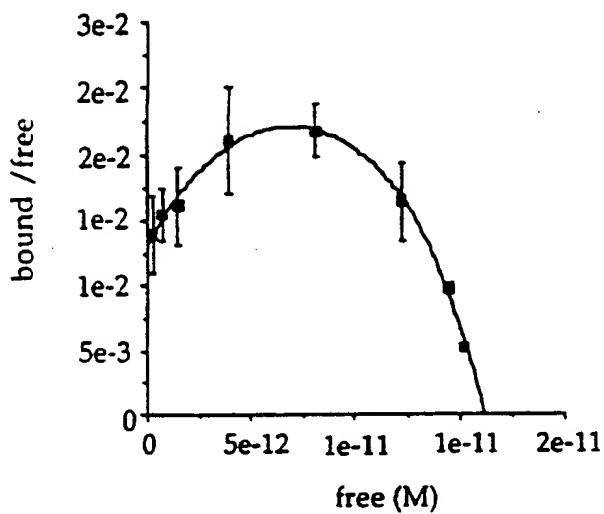
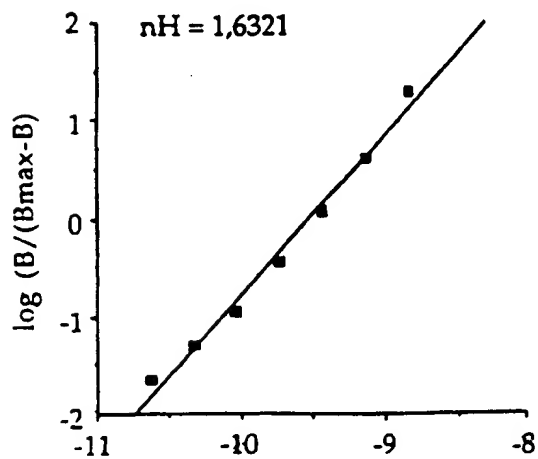
1. An isolated receptor which binds glial cell line-derived neurotrophic factor (GDNF), said receptor comprising at least one polypeptide having a
5 molecular weight selected from the group consisting of polypeptides of about 55kD, 70kD, 135kD, and 300kD molecular weight, as determined by SDS-PAGE on 4-20% gradient gels.
2. A competitive assay for identifying compounds which bind to GDNF
10 receptors comprising
 - a) incubating said compounds with cells which express c-RET receptors in the presence of an excess of labeled GDNF;
 - b) measuring the amount of labeled GDNF bound to said cells; and
 - c) comparing amount labeled GDNF bound to said cells to that of controls
15 not incubated with said compounds.
3. The method of claim 2 wherein the cells are selected from the group consisting of NB2/a, MN-1, and PC12 cells.
- 20 4. The method of claim 2 wherein the labeled GDNF is ¹²⁵I-GDNF.
5. A competitive assay for identifying compounds which bind to isolated GDNF receptors comprising
 - a) incubating said compounds with isolated c-RET receptors in the presence
25 of an excess of labeled GDNF;
 - b) measuring the amount of labeled GDNF bound to said receptors; and
 - c) comparing amount labeled GDNF bound to said receptors to that of controls not incubated with said compounds.
- 30 6. The method of claim 5 wherein the receptors are polypeptides which bind GDNF selected from the group consisting of polypeptides about 55kD, 70kD, 135kD, 155kD, and 300kD molecular weight.

7. The method of claim 6 wherein the polypeptide is about 155kD molecular weight.
8. The method of claim 5 wherein the isolated receptor is c-RET.
- 5 9. The method of claim 5 wherein the labeled GDNF is ¹²⁵I-GDNF.
10. A method for identifying compounds which are GDNF homologs comprising
- 10 a) incubating said compounds with cells which express c-RET receptors; and
- b) determining whether said compound effects tyrosine phosphorylation.
11. The method of claim 10 wherein said cells are selected from the group consisting of PC12, MN-1, and NB2/a.
- 15 12. A method for identifying compounds which are GDNF homologs comprising
- a) incubating said compounds with cells which express c-RET receptors;
- 20 and
- b) determining whether said compounds effect an increase in *c-fos* mRNA levels.
13. The method of claim 12 wherein said cells are selected from the group consisting of PC12, MN-1, and NB2/a.
- 25 14. A method for identifying compounds which are GDNF homologs comprising
- a) incubating said compounds with cells which express c-RET receptors
- 30 under non-permissive conditions for said cells; and
- b) determining the number of surviving cells as compared to controls not incubated with said compounds.

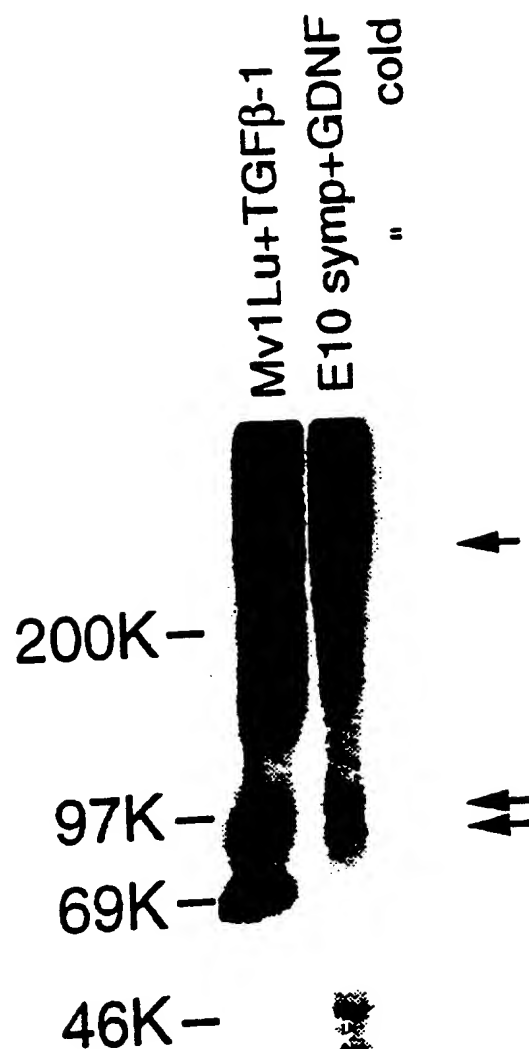
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15. The method of claim 14 wherein said cells are selected from the group consisting of PC12, MN-1, and NB2/a.
16. A method for identifying compounds which are GDNF analogs comprising
5 a) incubating said compounds with cells which express c-RET receptors in the presence of concentrations of GDNF effective for phosphorylating tyrosine; and
b) determining whether said compounds effect a decrease in the tyrosine phosphorylation as compared with controls not incubated with said compounds.
- 10 17. The method of claim 16 wherein said cells are selected from the group consisting of PC12, MN-1, and NB2/a.
18. A method for identifying compounds which are GDNF analogs comprising
15 a) incubating said compounds with cells which express c-RET receptors in the presence of concentrations of GDNF effective for increasing *c-fos* mRNA levels; and
b) determining whether said compounds effect a decrease in *c-fos* mRNA levels as compared with controls not incubated with said compounds.
- 20 19. The method of claim 18 wherein said cells are selected from the group consisting of PC12, MN-1, and NB2/a.
20. A method for identifying compounds which are GDNF analogs comprising
25 a) incubating said compounds with cells which express c-RET receptors under non-permissive conditions for said cells in the presence of amounts of GDNF effective for cell survival; and
b) determining the number of surviving cells as compared with controls not incubated with said compounds.
- 30 21. The method of claim 20 wherein said cells are selected from the group consisting of PC12, MN-1, and NB2/a.

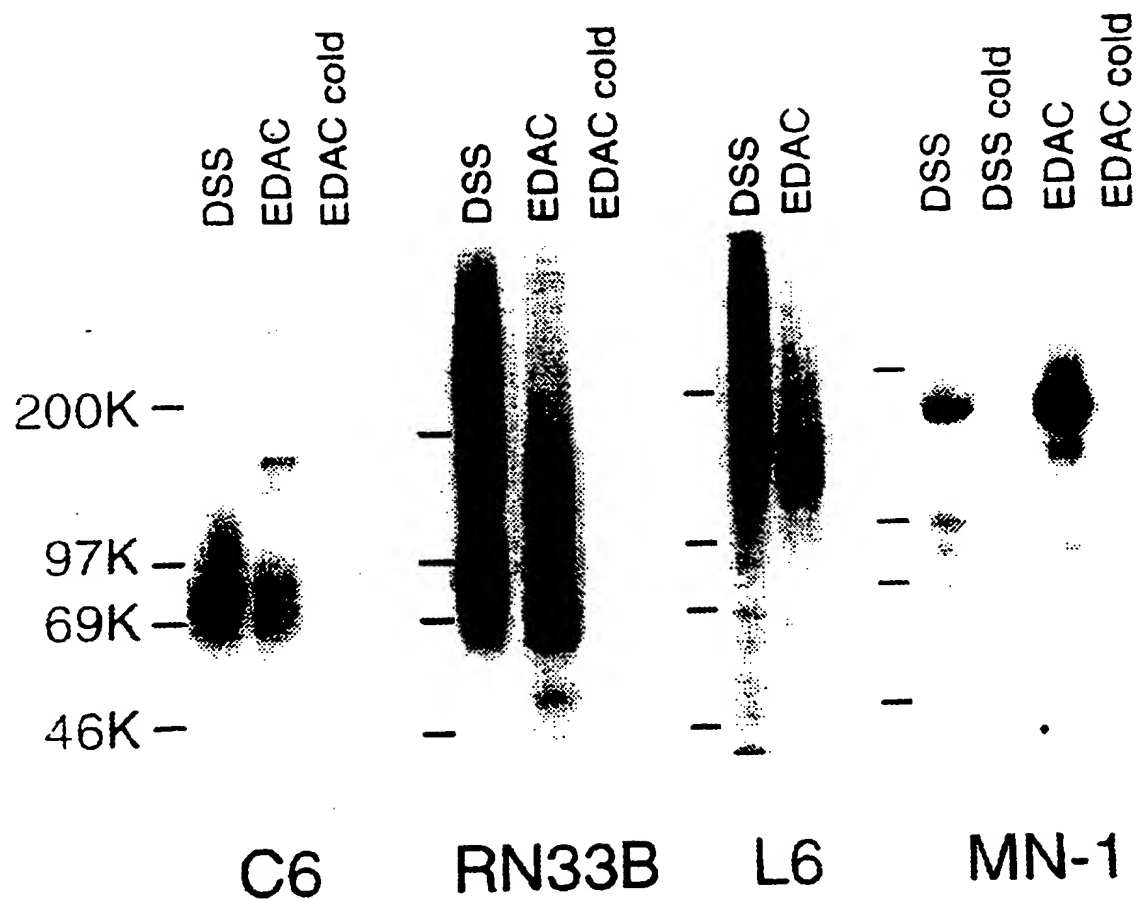
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*FIG. 1A**FIG. 1B**FIG. 1C*

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**FIG. 2**

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**FIG. 3**

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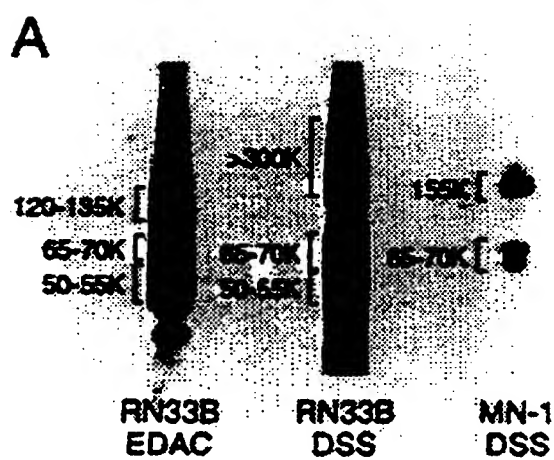


FIG. 4A

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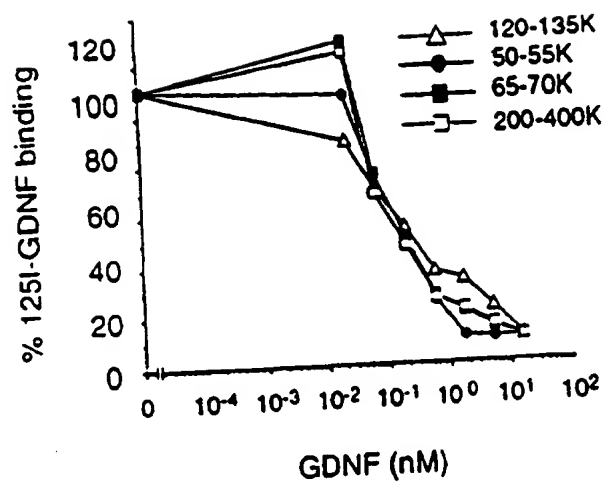


FIG. 4B

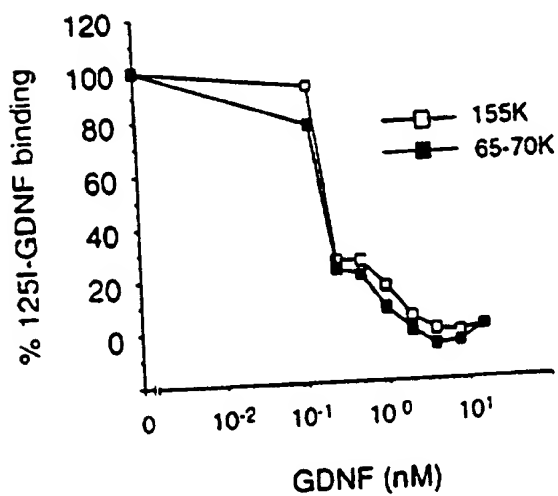


FIG. 4C

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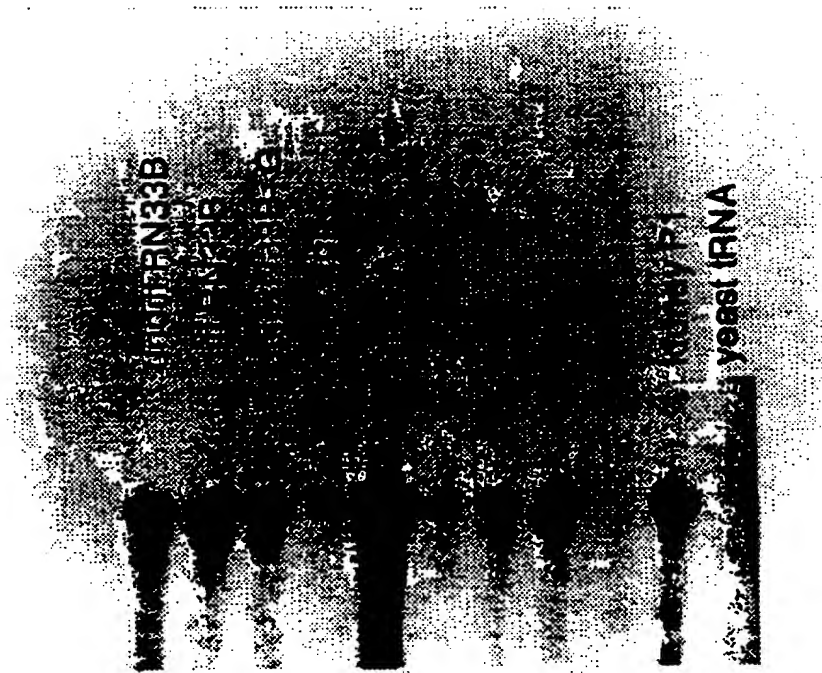
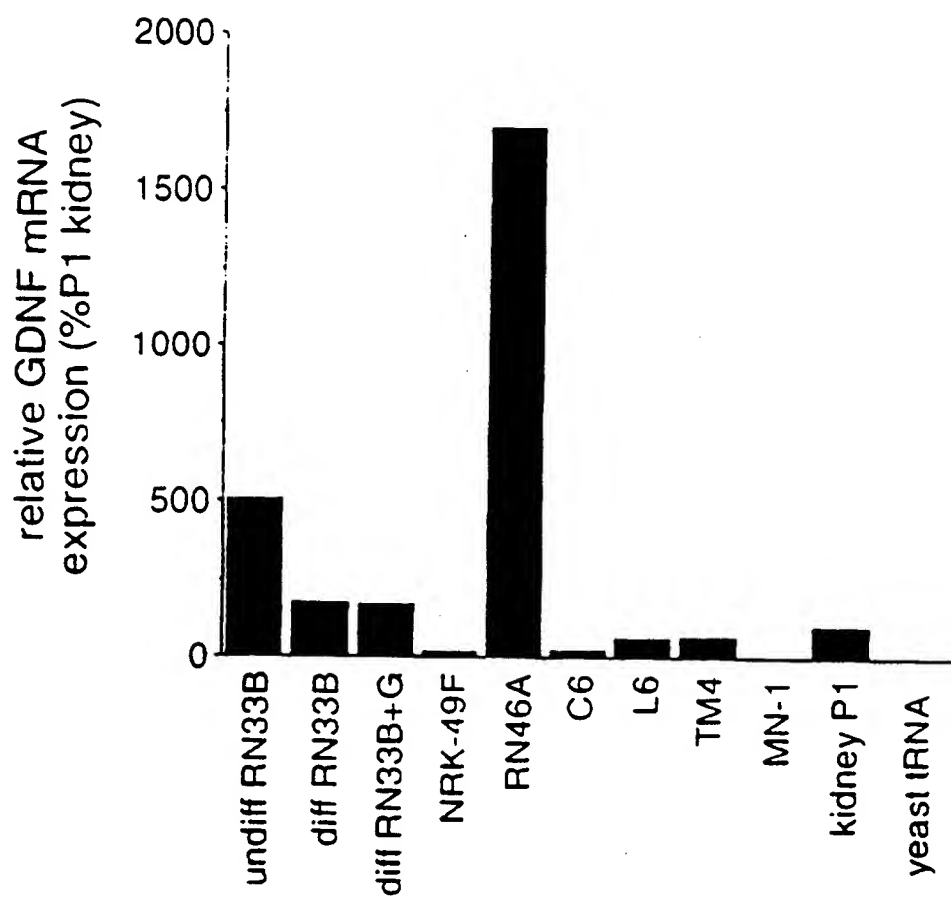


FIG. 5A

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*FIG. 5B*

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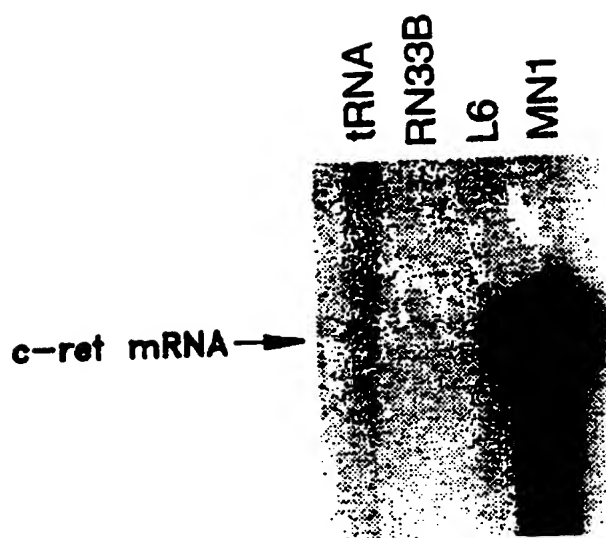
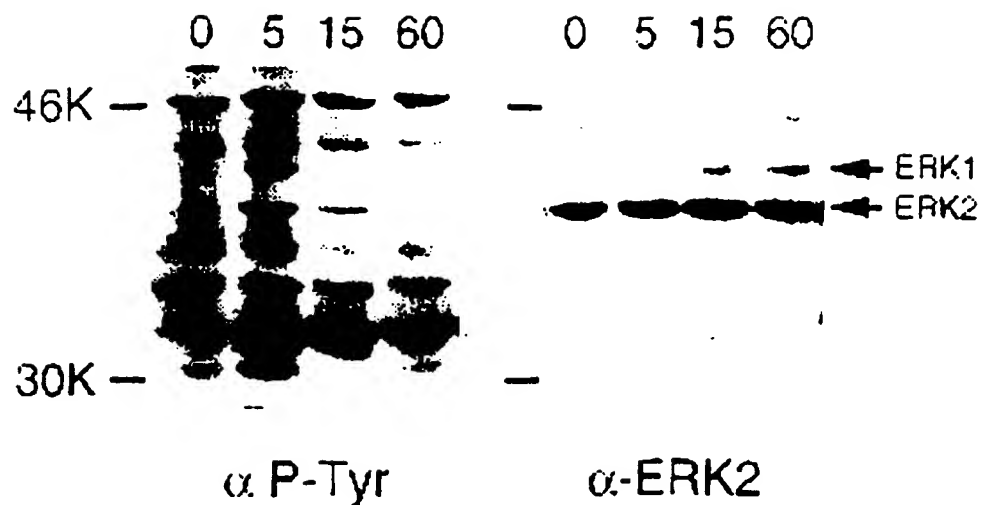
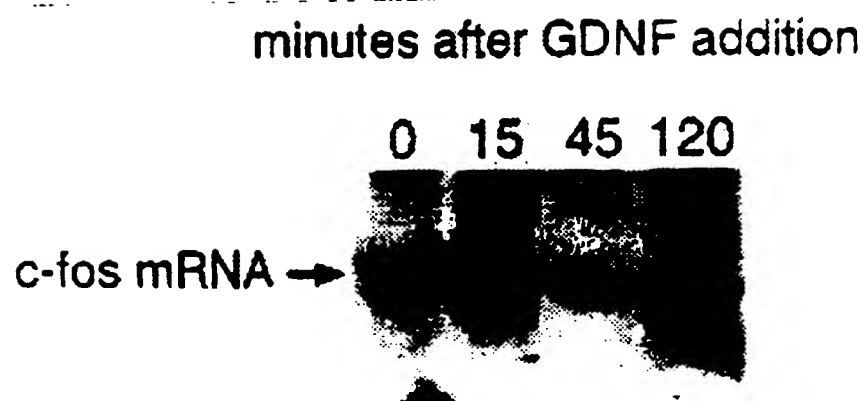
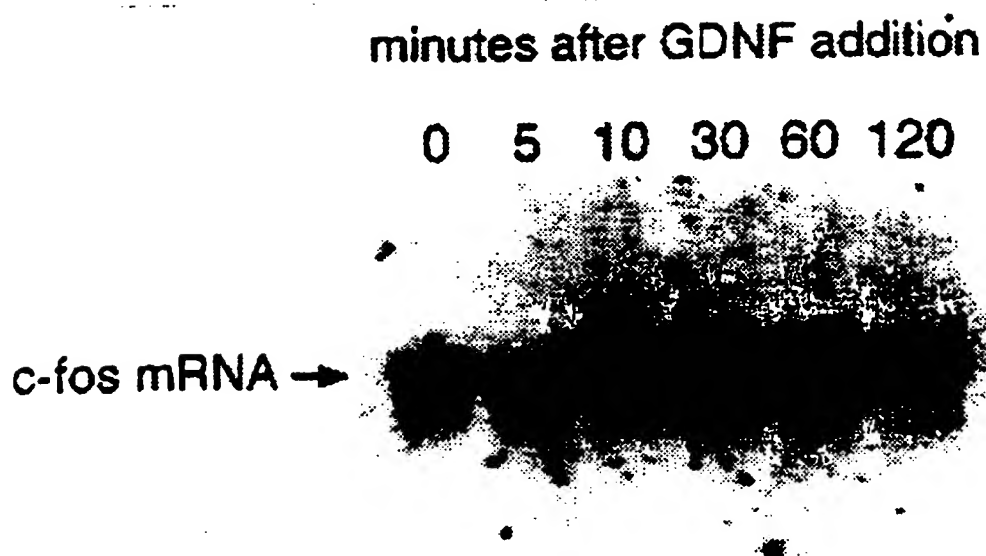


FIG. 6

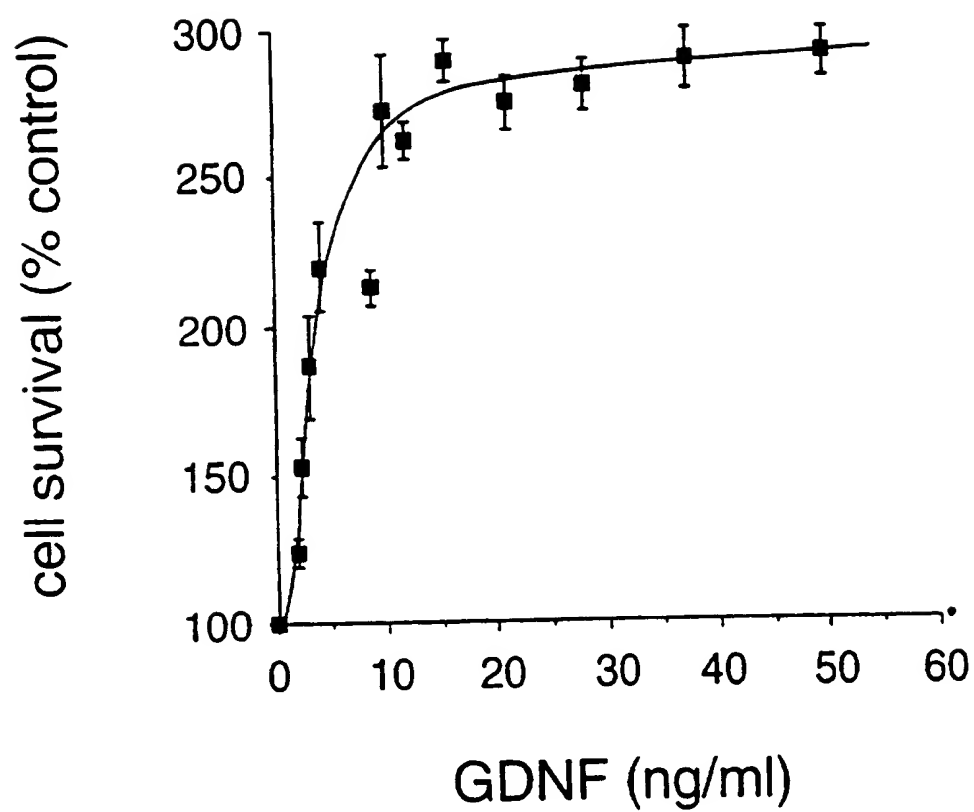
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**FIG. 7A****FIG. 7B**

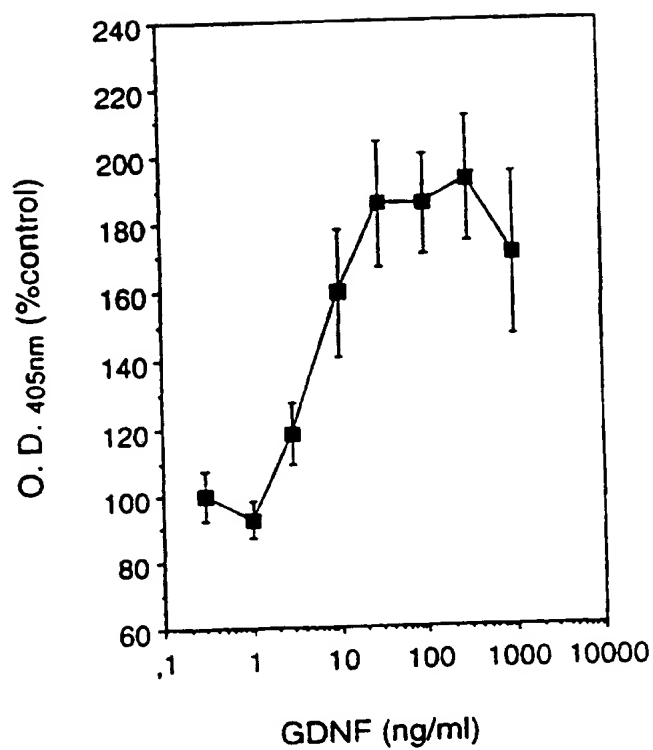
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*FIG. 8A**FIG. 8B*

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*FIG. 9*

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*FIG. 10A*

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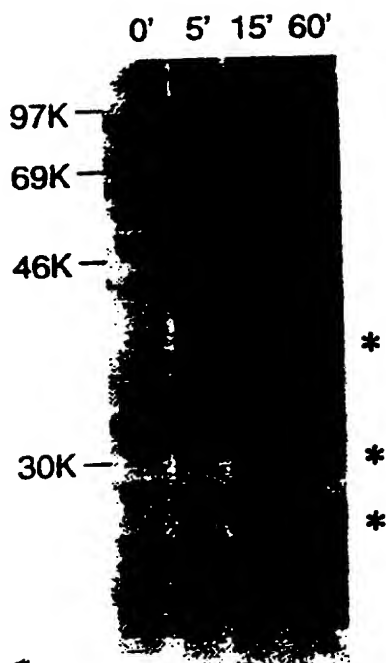


FIG. 10B

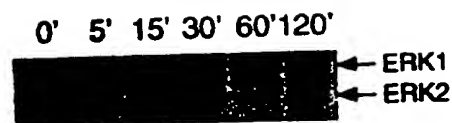


FIG. 10C

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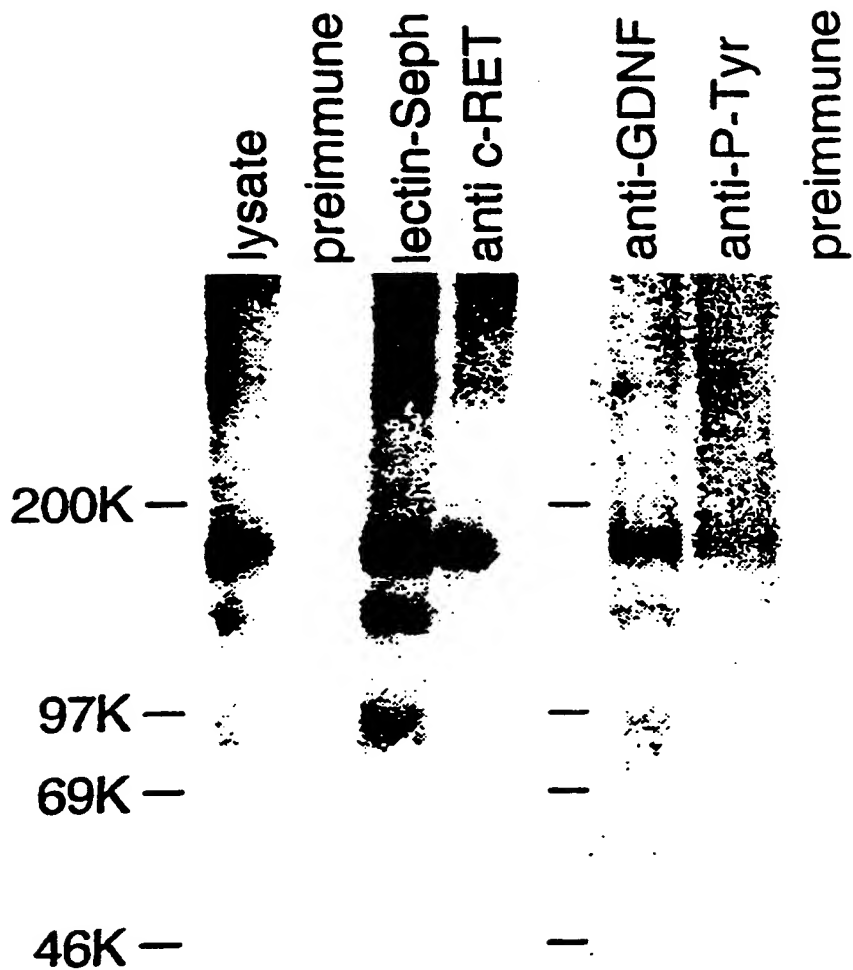


FIG. 11A

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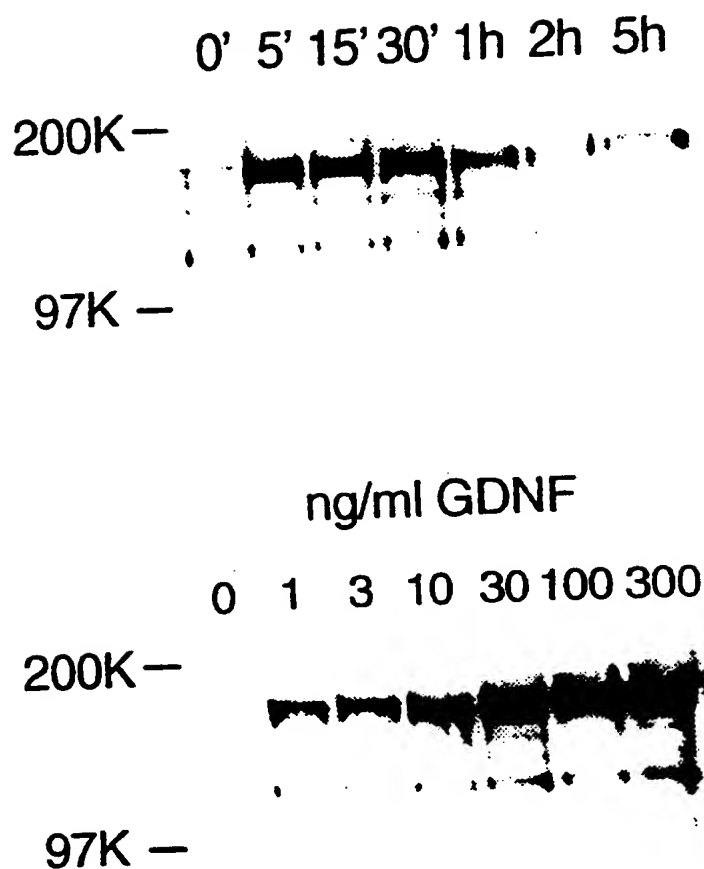


FIG. 11B

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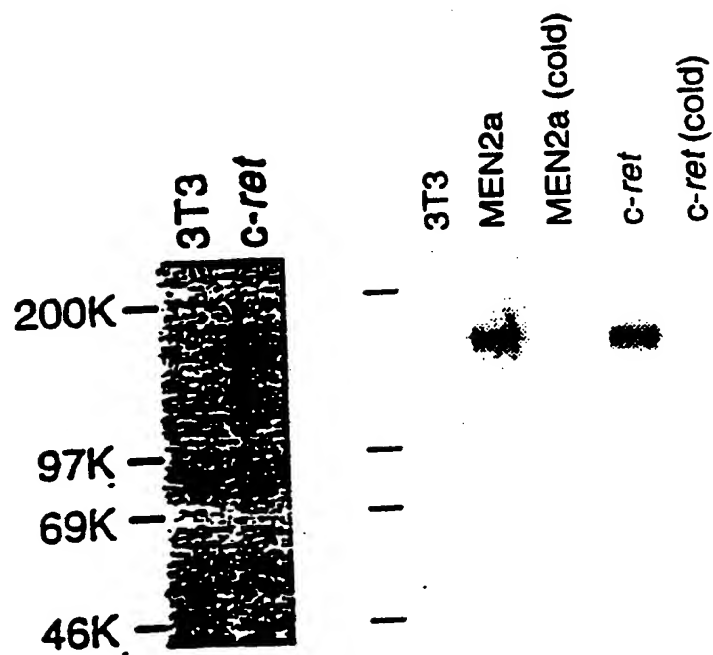
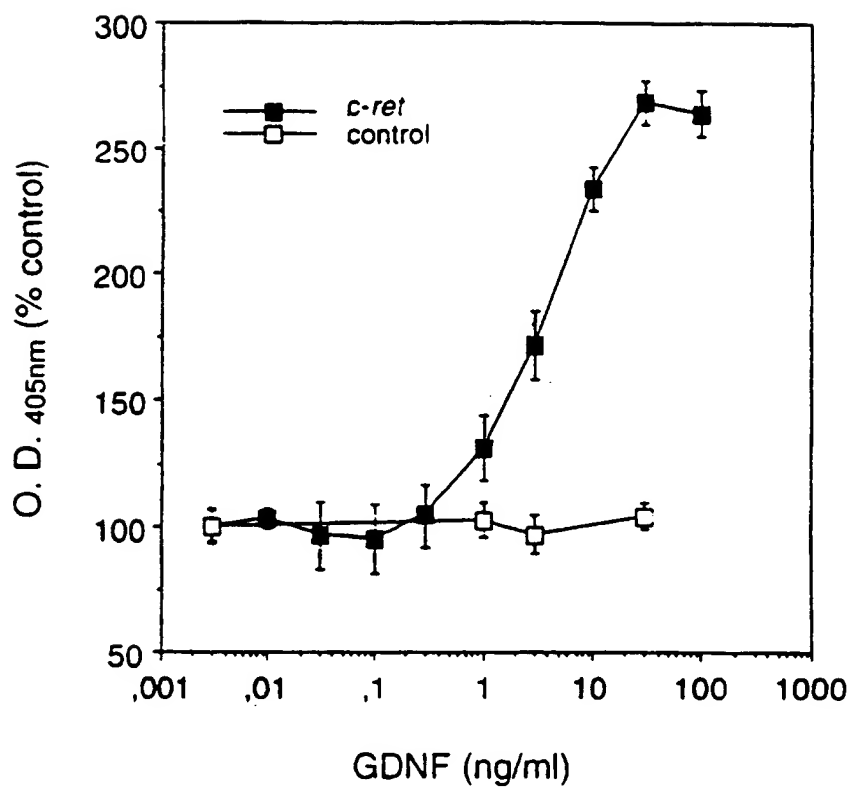
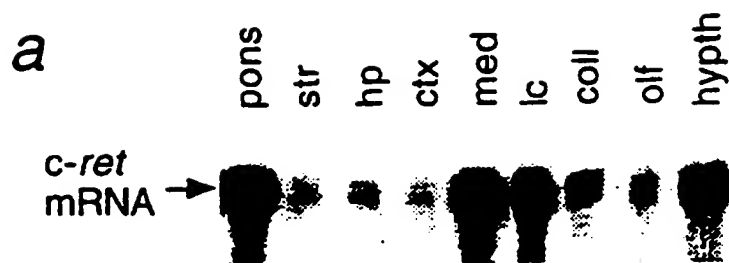
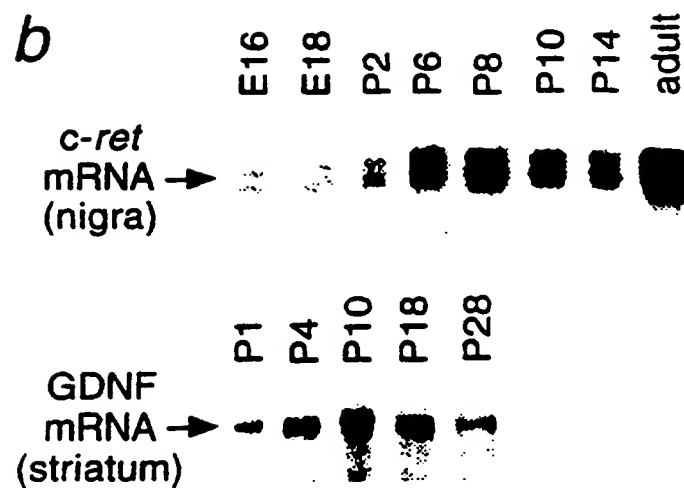


FIG. 12A

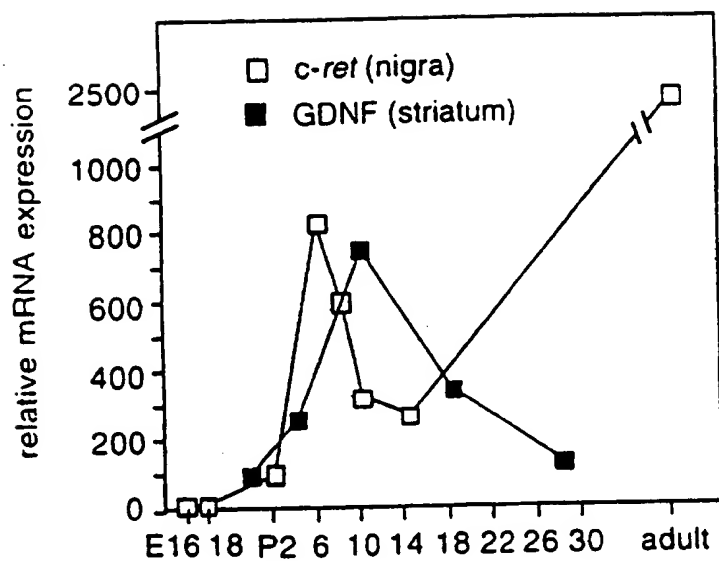
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*FIG. 12B*

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*FIG. 13A**FIG. 13B*

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*FIG. 13C*

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FIG. 14A

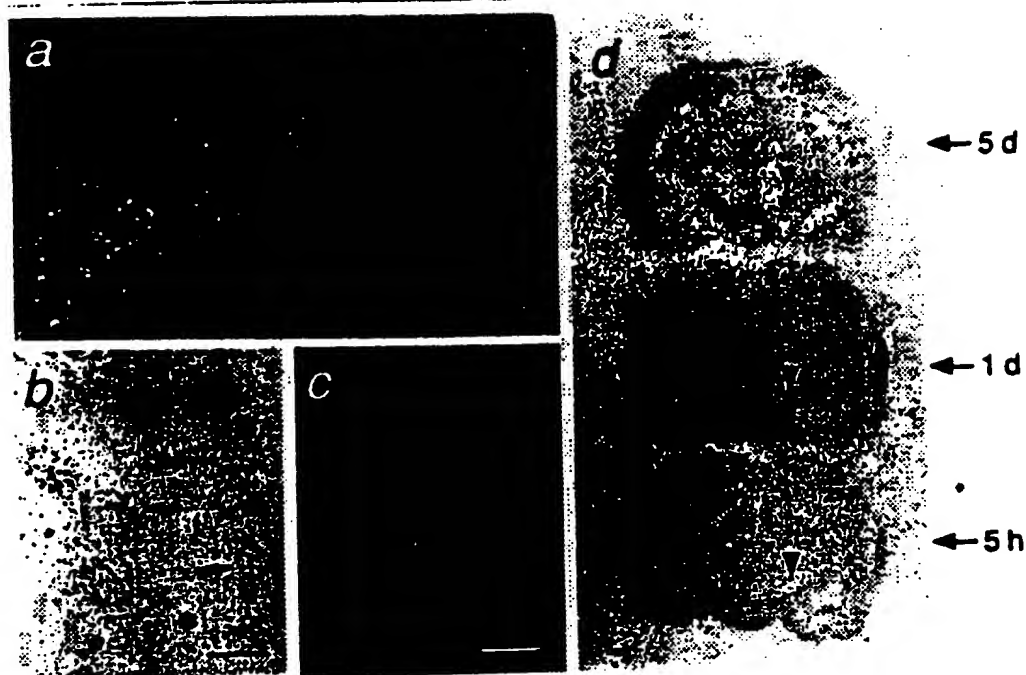


FIG. 14B *FIG. 14C* *FIG. 14D*

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FIG. 14E

FIG. 14F

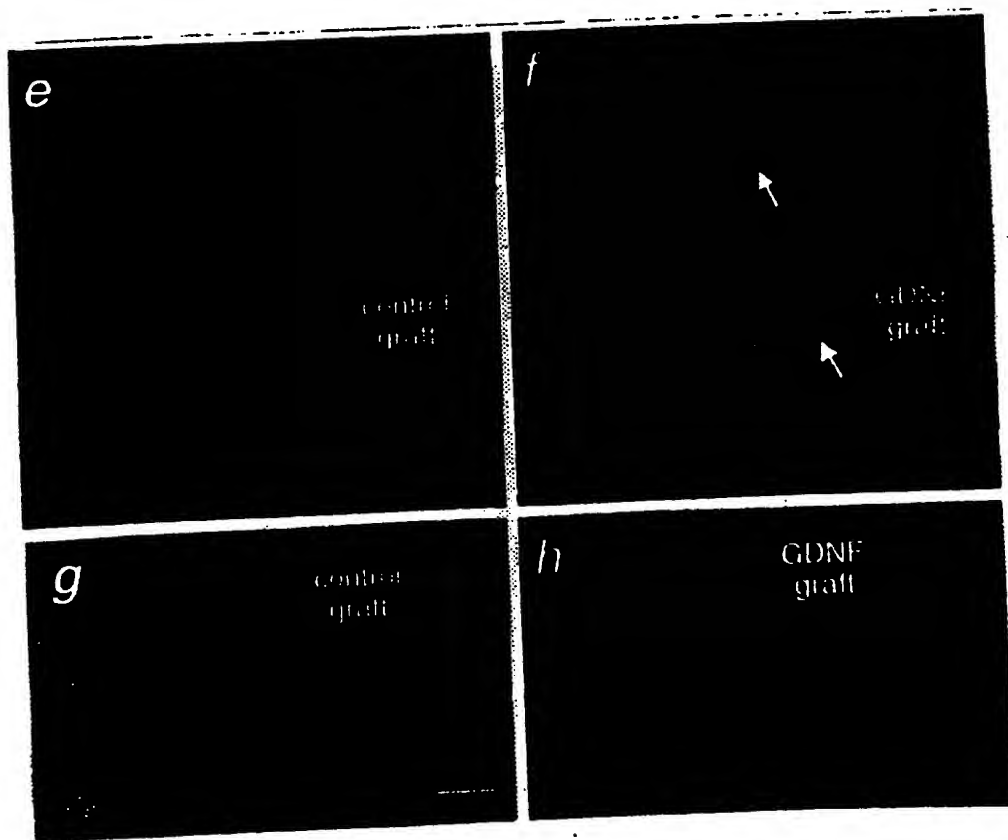


FIG. 14G

FIG. 14H

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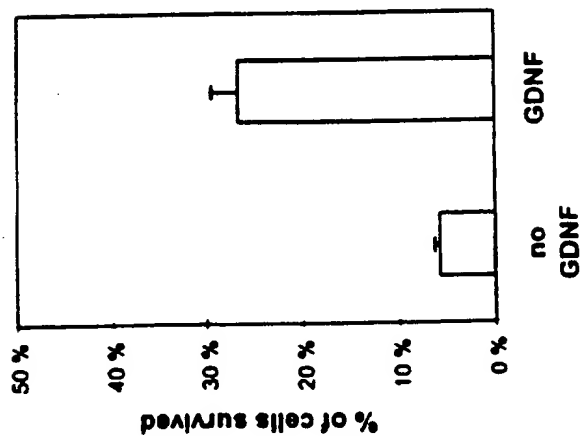
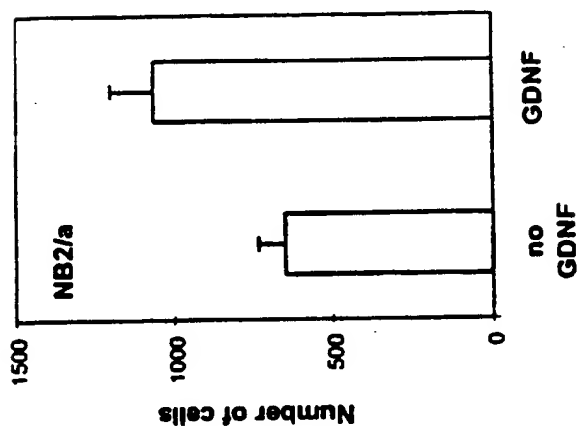
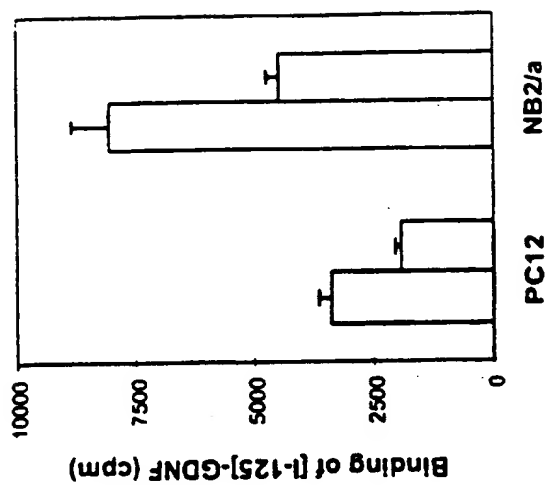


FIG. 15C

FIG. 15B

FIG. 15A

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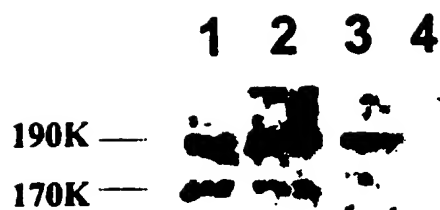


FIG. 16

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COLD GDNF

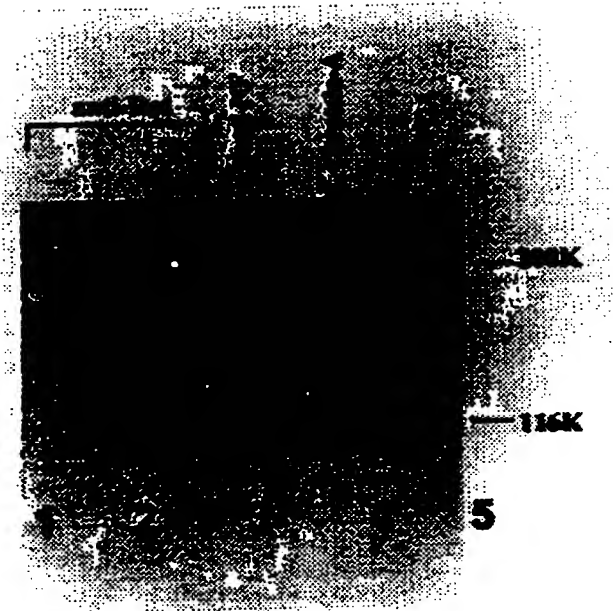
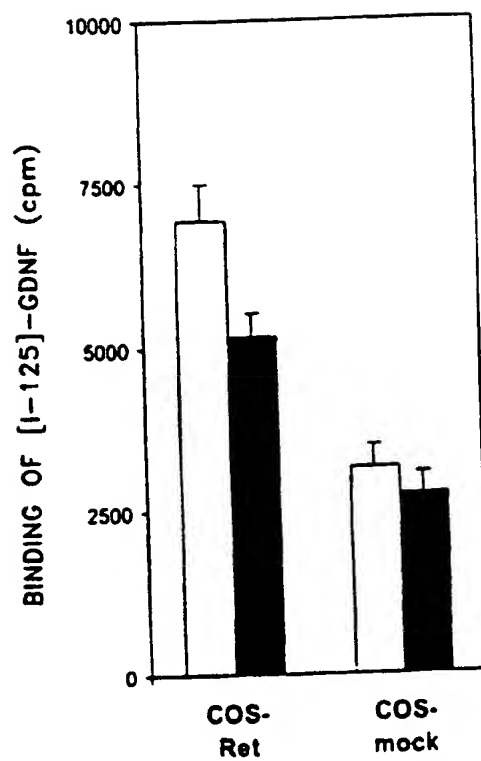


FIG. 17

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*FIG. 17B*

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*FIG. 18*

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FIG. 19A



FIG. 19B



FIG. 19C

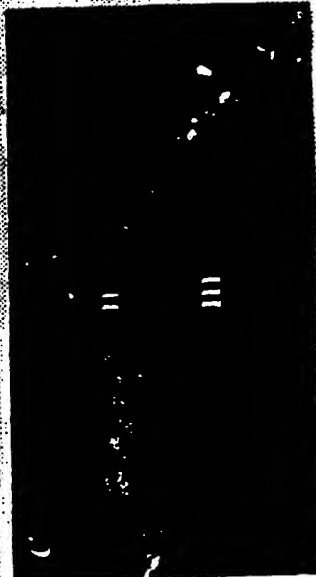


FIG. 19D



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FIG. 19F

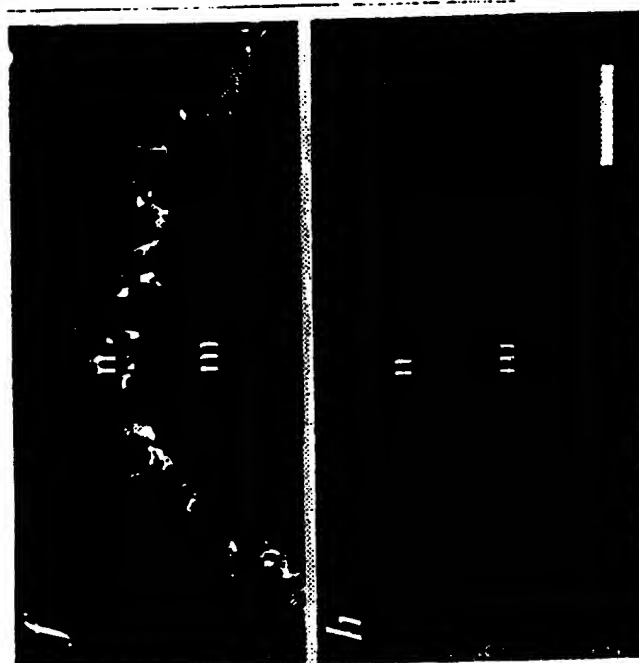


FIG. 19E

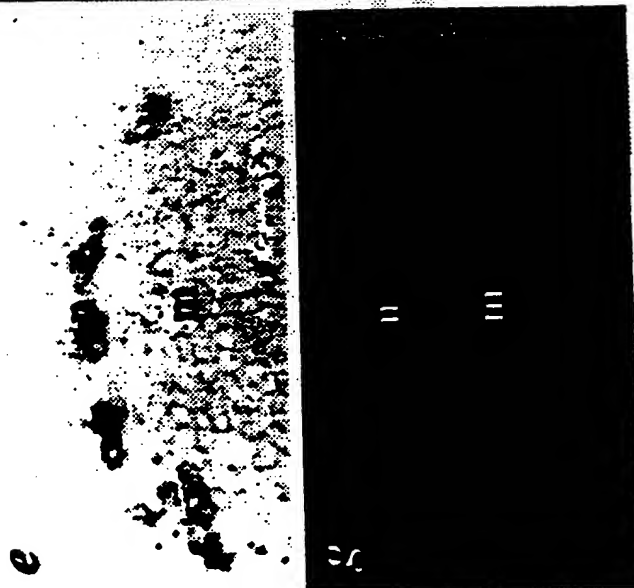


FIG. 19H

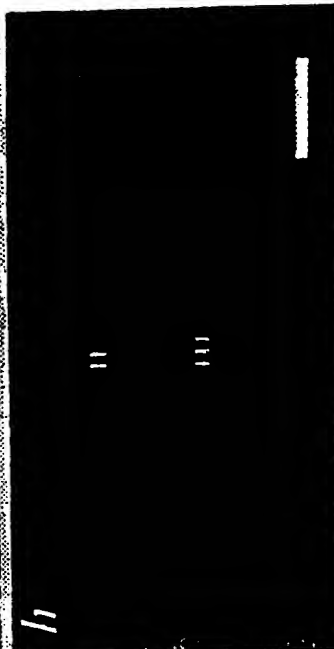
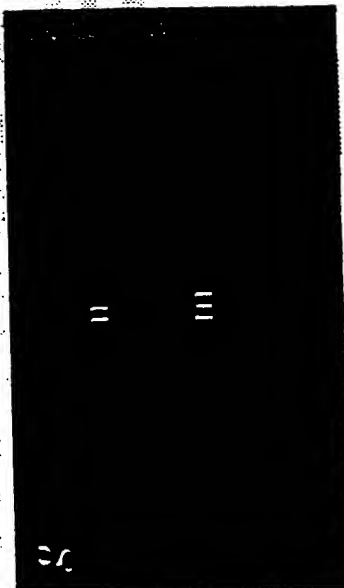


FIG. 19G



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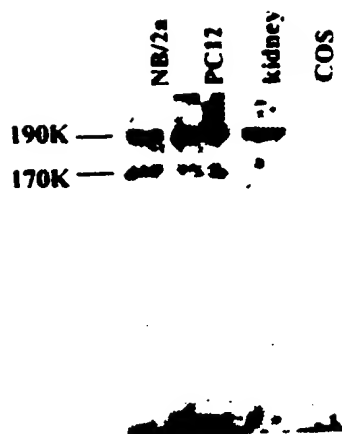


FIG. 20A

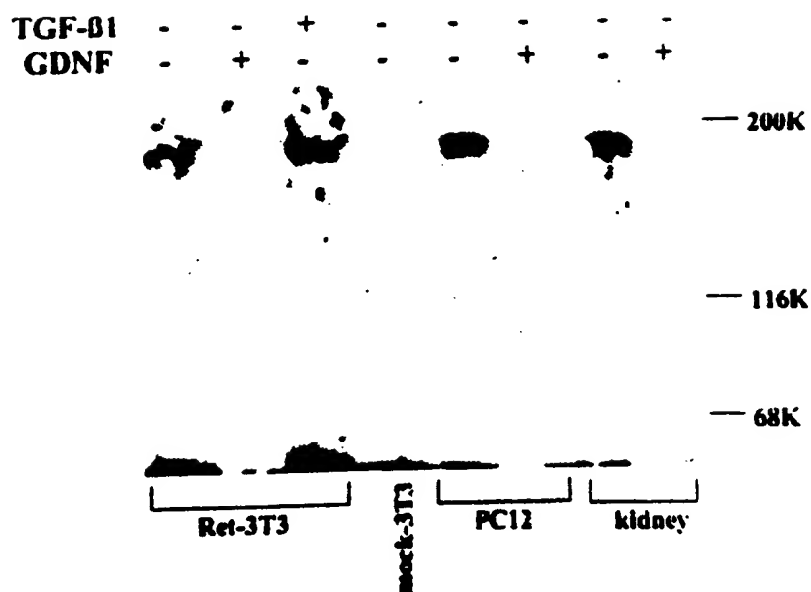
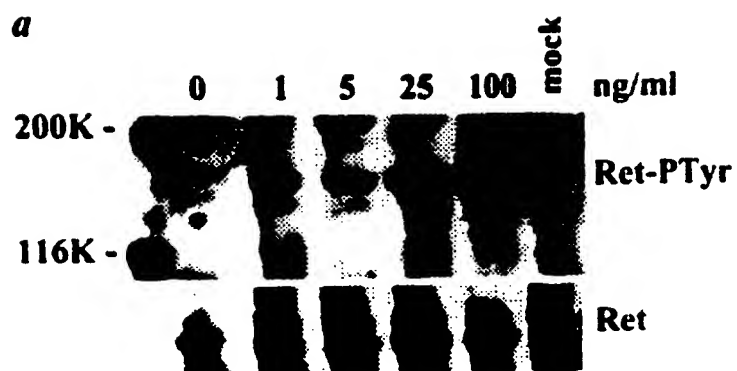
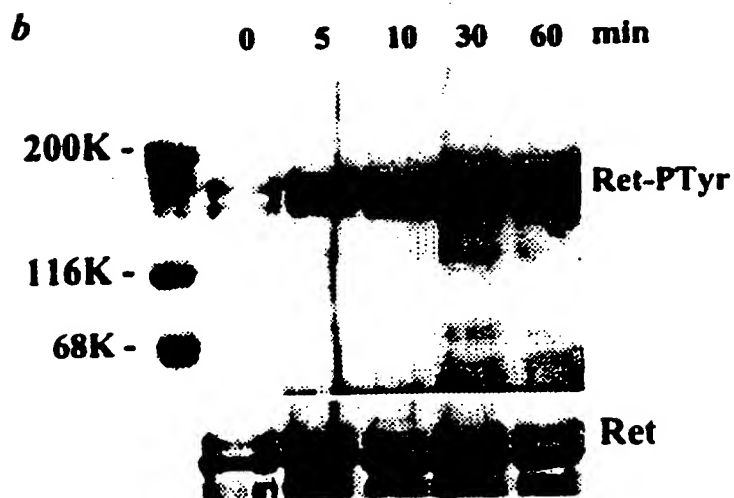
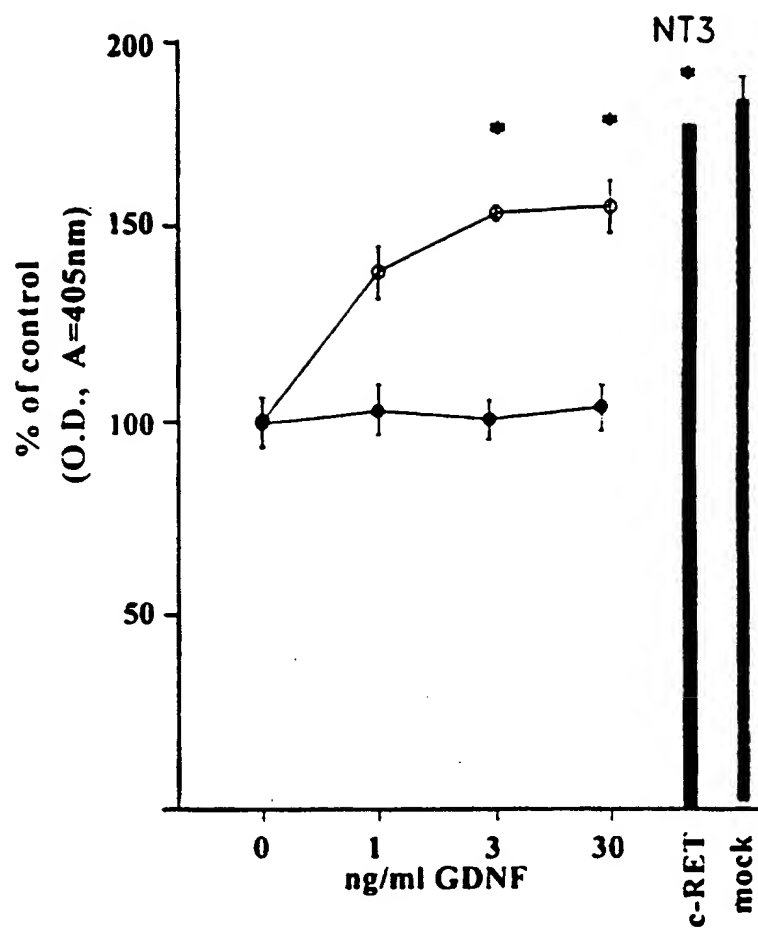


FIG. 20B

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*FIG. 21A**FIG. 21B*

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*FIG. 22*

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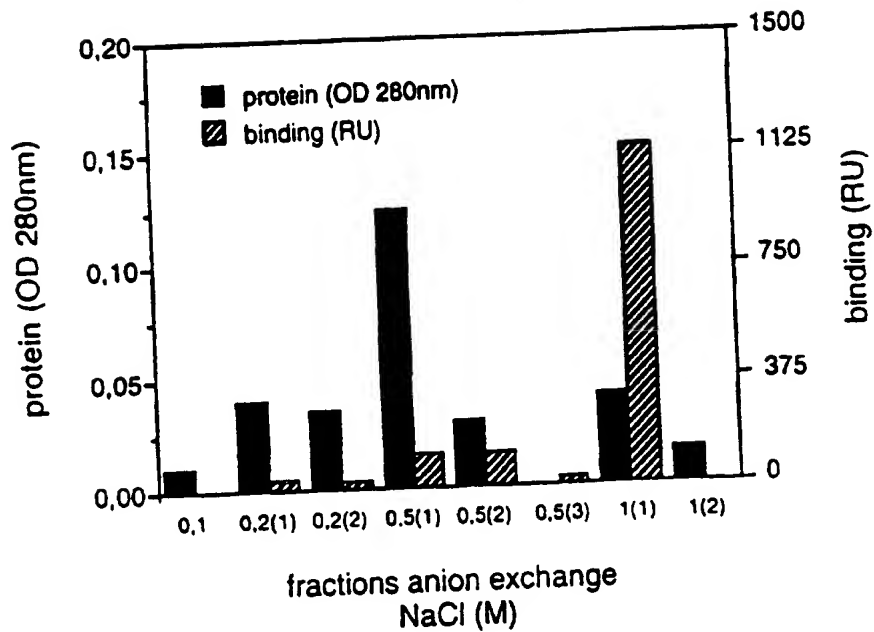


FIG. 23A

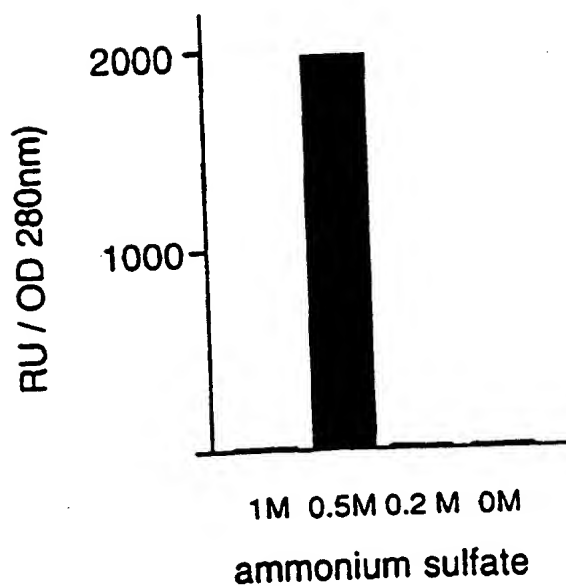


FIG. 23B

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/18197

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐

The additional search fees were accompanied by the applicant's protest.

☐

No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/18197

A. CLASSIFICATION OF SUBJECT MATTER: IPC (6):

C07K 14/705; G01N 33/566

A. CLASSIFICATION OF SUBJECT MATTER: US CL :

530/350; 435.7.2; 436/87, 501

B. FIELDS SEARCHED Minimum documentation searched Classification System: U.S.

530/350; 435.7.2; 436/87, 501

B. FIELDS SEARCHED Electronic data bases consulted (Name of data base and where practicable terms used):

CAPLUS, Medline, WPIDS, FEDRIP, EMBASE, APS
Search terms: GDNF, receptor, c-RET, phosphorylation, tyrosine, c-fos, assay

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1.

Group I, claims 1 and 5-9, drawn to an isolated receptor that binds GDNF and an assay for identifying compounds that bind to isolated GDNF receptors.

Group II, claims 2-4, drawn to a method of identifying compounds that bind to GDNF receptors, using cells that express c-RET receptors.

Group III, claim 10-21, drawn to a method of identifying GDNF homologs and analogs, using cells that express c-RET receptors.

The inventions listed as Groups I-III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The special technical feature of Group I is to use the isolated GDNF receptor. The special technical feature of Group II is to identify compounds that bind to c-RET receptors. The special technical feature of Group III is to identify GDNF analogs using cells that express c-RET receptors. Groups I and (II-III) are not linked by a single inventive concept because the methods of (II-III) do not use the isolated receptor of Group I, but use cells that express the receptor. The methods of Groups II and III involve different steps, issues, and objectives. Note that PCT Rule 13 does not provide for multiple methods within a single application.

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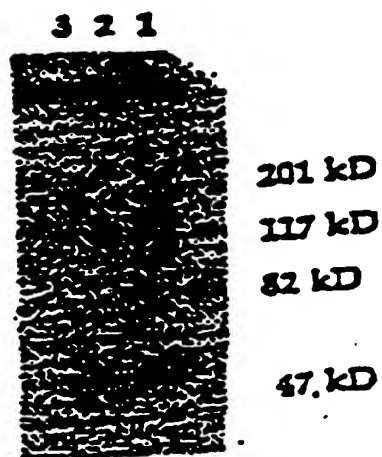


FIG. 24

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/18197

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	TRUPP et al. Peripheral Expression and Biological Activities of GDNF, a New Neurotrophic Factor for Avian and Mammalian Peripheral Neurons. The Journal of Cell Biology. July 1995, Volume 130, Number 1, pages 137-148, see entire document.	1-21
Y	TREANOR et al. Characterization of GDNF Binding to Putative GDNF Receptor. Society for Neuroscience. 1995, Volume 21, page 1301, Abstract No. 515.14, see entire abstract.	1-21

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	* T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
* A document defining the general state of the art which is not considered to be of particular relevance	* X	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
* E earlier document published on or after the international filing date	* Y	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
* L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	* Z	document member of the same patent family
* O document referring to an oral disclosure, use, exhibition or other means		
* P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

18 FEBRUARY 1997

Date of mailing of the international search report

17 MAR 1997

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks

Authorized officer

Handwritten signature